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REFLEX GRASPING AND GROPING

ITS SIGNIFICANCE IN CEREBRAL LOCALIZATION *

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The reflex grasping produced in the infant by introducing the finger into its palm is a phenomenon too well known to deserve description It disappears as the child develops, probably at about the age of 1 year, although the exact time is variable This phenomenon may appear in later life as a manifestation of disease of the brain either focal or general In certain cases it is of definite localizing value, pointing accurately to the contralateral frontal lobe

Only within the past year has the symptom evoked much attention or discussion, although its nature seems to have been recognized by Janischewsky[1] as much as twenty years ago His extensive contribution last year, appearing almost simultaneously with those of Schuster,[2] and of Adie and Critchley,[3] has served to focus the attention of neurologists on what appears to be a valuable sign in cerebral localization There is abundant evidence, however, that the phenomenon has been repeatedly encountered in the past, and even described under a variety of names Liepmann,[4] in his work on apraxia, frequently encountered "tonic perseveration and clonic perseveration" terms that Wilson and Walshe[5] criticized, substituting therefor "tonic innervation" and "clonic innervation" Adie and Critchley find "forced grasping and groping" most expressive It seems most clearly related to the prehensile reflex in the infant, in whom both tonic and clonic forms may be elicited, and in whom the same difficulties in the voluntary relaxation of the grasp may be observed even during the second year

The elemental part of the symptom or sign is elicited by stroking the thenar eminence or by inserting the fingers into the palm of the patient and rubbing them back and forth, at the same time pulling outward on the flexed fingers We believe that this simple test should be performed as a routine in the neurologic examination When the sign is positive there is a tendency to coordinated grasping by the thumb and fingers

The grasp is tonic, showing little variation in power over a period of several seconds The force is usually increased when the patient attempts to let go or when the object is forcibly withdrawn from the hand Sometimes the grasp is so strong that the patient's head and shoulders may be raised from the bed by traction on the seized object At times the contraction becomes clonic, particularly if the grasp is avoided and the object pressed against the flexed finger tips When the reflex is well marked, the patient himself may state that when he grasps an object he is unable to let go in spite of strenuous efforts to open the hand

In advanced cases not only is there reflex grasping of every object that comes in contact with the palm but in addition there may be involuntary groping, so that a touch on the back of the hand or on the arm or even on the face may cause the hand to follow immediately the exciting stimulus and to take hold Nurses are hampered in caring for such patients (e g , case 3) on account of the tenacity of the grip on the bedclothes or on the chemise Such patients may even have considerable difficulty in feeding themselves or in being fed because of the tendency on the part of the affected hand to grasp the food or the utensil or the hand of the nurse and pull it away from the mouth Not infrequently, apparently without any stimulus, these patients will raise their arms and wave their hands about in the air, opening and closing the fingers, and groping, then possibly bringing their hands to the face before subsiding

Phenomena similar to reflex grasping have been recognized in the lower extremities and even in the jaw A light tap at the base of the infant's toes will cause them to fold over the object as the fingers do, although the reaction is only momentary This phenomenon was elicited in cases 3, 4 and 5 Wilson and Walshe noted "tonic innervation" in the extended leg when the patient was told to flex it, and vice versa Janischewsky publishes a striking picture of a patient with a towel grasped between the teeth, he terms this the "bulldog reflex" He noted that pressure on the teeth was necessary We have observed tonic closure of the jaws when an attempt is made to force open the mouth

For pathologic grasping and groping to be manifested, it seems necessary that the corticospinal tract for the hand shall be relatively intact In two patients of Wilson and Walshe the symptoms disappeared when ordinary hemiplegia followed the removal of meningeal tumors Other instances of disappearance of the symptom with extension of the disease and the production of total hemiplegia have been observed Quite often however, paralysis of the lower extremity is found

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* From St Elizabeth's Hospital and from the United States Naval Hospital respectively

[1] Janischewsky A. Deutsche Ztschr f Nervenh. **102** 177 195 1928

[2] Schuster P Autoptische Befunde bei Zwangsgreifen und Nachgreifen Ztschr f d ges. Neurol u Psychiat. **108** 715 734 1927

[3] Adie W J and Critchley M Forced Grasping and Groping Brain **50** 142 (June) 1927

[4] Liepmann cited by Wilson and Walshe (footnote 5)

[5] Wilson and Walshe The Phenomenon of Tonic Innervation and Its Relation to Motor Apraxia Brain **37**: 198 1914

of digipuratum received in July, 1913, were a this laboratory in July, 1920, and found ıan half as active as a corresponding amount specimen, then called digitan We examined pecimen in ampules of unknown age but more than 10 years old, and found it about as active as a corresponding amount of the cimen of digitan, but we had enough for only

cimen of tincture of digitalis in ampules from he air had been removed before sealing, and ing 44 per cent of alcohol, was examined in this ory in March, 1918 After an interval of about ars, it had apparently decomposed to the extent out 45 per cent, but the material available was ıent for a test on only one animal

specimen of fluidextract of digitalis obtained from R Squibb & Sons in 1913, at which time it was east 30 years old, was examined by Hatcher and gleston and later it was used clinically It was then s active than the average fresh fluidextract of good ality It was examined by one of us in 1919, at hich time the activity was the same as that found by latcher and Eggleston in 1913 Our examination does ıot indicate that it has undergone any deterioration luring the interval of sixteen years following its first examination, but there is a possibility that some evaporation occurred, as the bottle contained only about 2 cc

An active specimen of fluidextract of digitalis obtained from the H K Mulford Company and examined by one of us in 1913 was kept in a cork-stoppered bottle, and in 1919 its activity was 83 per cent of the original Later it was transferred to a glass-stoppered bottle, and the result of our examination indicates that it lost about 32 per cent of its original activity in sixteen years

A specimen of tincture of digitalis prepared by one of us in 1920, which deteriorated rapidly during the first six months, was examined again after an interval of nine years It had changed very little after the second examination and it is now only a little less active than it was six months after it was prepared It has been kept throughout the period in a cork-stoppered and only partly filled flint bottle

A specimen of tincture made by one of us in 1913 lost about 27 per cent of its activity in sixteen years, another made in 1913 from the leaf then 25 years old with 50 per cent alcohol lost about 38 per cent of its activity in sixteen years and a very active tincture prepared in this laboratory in 1912 lost about one half of its activity in sixteen and one-half years

A tincture prepared by one of us in January, 1918, and tested at that time showed only slight deterioration after fifteen months but after eleven years and two months it had lost 35 per cent of its activity

An active specimen of tincture prepared by one of us in August 1922 was only about 14 per cent less active when examined after an interval of nearly seven years A similar change occurred in another tincture in four years and seven months

Four specimens of digitol were tested The first digitol aqueous has been discussed One specimen had been kept in an ordinary glass bottle for ten and one-half years, another was kept for an unknown period of time in a sealed 'vacule" so called because the air had been removed by a filter pump before the container was sealed one specimen tested by the manufacturers, Feb 8, 1929, was tested by us ten weeks later Assuming that all were originally of uniform activity and that the most recent had not deteriorated, that kept in an ordinary bottle lost ab in ten and one-half years, that in the 70 per cent alcohol lost 42 per cent of its activity shows that such elaborate precautions for the removal of air are unnecessary A corked 1-ounce bottle filled in the ordinary way contains about 1 mg of atmospheric oxygen, and there is no reason to suppose that it has any perceptible influence on the decomposition of the active constituents of digitalis

W A Puckner has long insisted that the labels of proprietary preparations which are subject to deterioration should bear the date of preparation or that after which the activity cannot be guaranteed Our results strongly support that contention and we believe that manufacturers should date all such preparations

A tincture of digitalis containing 65 per cent of alcohol was received in January, 1923, at which time it was said to be 50 per cent more active than the official tincture When examined after six years, it was about one-third less active than the average official tincture Hence, it had apparently undergone decomposition to the extent of about 50 per cent Two specimens of tincture of digitalis prepared by E R Squibb & Sons, April 6, 1911, were received in this laboratory in 1913, and examined by us after an interval of eighteen years following their preparation The first of these was about one-fourth weaker than an average fresh tincture of good quality The other had been made from the same specimen of digitalis but which had been treated with magnesium carbonate to remove calcium salts and to neutralize acidity Its activity was equal to about 60 per cent of that of the first and it was only about half as active as an average fresh tincture of good quality A tincture prepared in 1913, and containing 44 per cent of alcohol, was a little less than half as active as an average fresh tincture

Two specimens of purified tincture prepared by one of us with 60 per cent alcohol in 1921, and one in 1922 and standardized at those times, retained their activity unimpaired during seven years, and an ether-soluble fraction of digitalis principles in 60 per cent alcohol also retained its activity unchanged during eight years The purified tincture consists of the so-called gitalin fraction of digitalis obtained by shaking an infusion with chloroform, distilling the chloroform, and dissolving the residue in 60 per cent alcohol A slightly different preparation of this type kept in 60 per cent alcohol lost about half its activity in seven years One weakly alcoholic solution became inert, another lost about half of its activity Our experiments with purified tinctures are not sufficiently numerous to permit of the general statement that all such tinctures are equally stable, but they point to the importance of strong alcohol as a factor in the stability of tincture of digitalis Other experiments, however, prove that strong alcohol is not the only factor, since the rate of decomposition in some strongly alcoholic tinctures was equal to that in others of low alcoholic content in which other factors were more favorable for stability The stability of these purified tinctures is of theoretical, rather than of practical, importance to the clinician because dried digitalis leaf is permanent and because the official tincture and the official infusion are sufficiently stable for all ordinary purposes

The results of our experiments and of those others cited show that properly dried digitalis leaf m be kept almost indefinitely without deterioration special process of drying or extraordinary skill is nec sary It may be kept in the form of the whole l in powder or in tablets associated with reflex grasping in the upper extremity on the same side

The relation of reflex grasping to apraxia was formerly considered close, but Wilson and Walshe in 1914 pointed out that they are not the same, although they may be, and frequently are, associated The relationship to myotonia, on the other hand, is quite distant, the two syndromes having in common only the tonic innervation

LOCALIZATION

Reflex grasping may occur in such transient states as the epileptic aftermath, cerebral concussion or minor cerebral infarction It may also occur in terminal conditions of unconsciousness associated with muscular rigidity We have observed it in cases of cerebral hemorrhage, uremic coma and tuberculous meningitis, and we are inclined to believe that in most, if not all, instances of carphology, tonic and clonic grasping will be found if the adequate stimulus is applied In such cases of unconsciousness, either transient or heralding the terminal event, reflex grasping appears to be a manifestation of partial "decerebration", it is bilateral and it does not have any especial localizing value, merely indicating a disturbed functional condition of the brain To be of value, consciousness must be preserved, or at least in the unconscious patient the sign must be unilateral

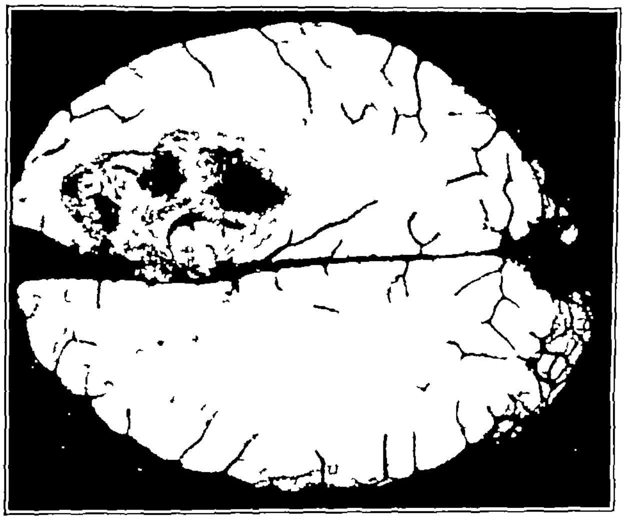
Fig 1 (case 1) —Glioma of right frontal lobe. In this case there was forced grasping on the left side.

It has been abundantly demonstrated that reflex grasping may result from focal lesions, and herein lies its localizing value Of the fifteen cases followed to necropsy by Schuster, twelve showed important lesions in the frontal lobe, while in the other three the lesions were more centrally located In two cases reported by Wilson and Walshe the meningeal tumors lay fairly close to the midline slightly anterior to the rolandic fissure Janischewsky has frequently found this area involved, but he believes that any interruption of the frontopontile projection tract that does not also involve the corticospinal tract for the hand may cause the appearance of reflex grasping

The same author has found this sign frequently in the various dystonias, especially in encephalitic parkinsonism We have observed the tightening of the grasp on attempting to straighten the fingers in advanced cases of parkinsonism and other dystonias, but we are disposed to attribute it rather to the general muscular hypertonus, since the contraction is momentary and since the grasp can be relaxed by the voluntary action of the patient, even though friction of the palm is continued

Most of our cases of bilateral reflex grasping have occurred in senile persons with cerebral arteriosclerosis, cases 3 4 and 5 being followed to necropsy We have observed the sign however in hemorrhagic pachymeningitis, in Little's disease and in a bilateral tuberculoma (case 2) Likewise in cases of unilateral grasping we attribute the sign in the majority of instances to cerebral infarction, usually in the anterior limb of the internal capsule, but we have also found it in infantile hemiplegia and in one case of glioma of the frontal lobe (case 1) In cases 3, 4 and 5 the thrombotic lesions were small but associated with them was rather profound atrophy of the whole frontal lobe on each side, the subcortical white matter having a porous gelatinous aspect that was rather widespread and probably depended on severe reduction in the number of nerve fibers and myelin sheaths Even the pons was affected by this reduction in the number of afferent elements, presenting a peculiar shriveled appearance

Of all the cases reported here, that of glioma of the frontal lobe (case 1) was the most significant, since the appearance of reflex grasping allowed us to make a preoperative diagnosis of tumor of the right frontal lobe

REPORT OF CASES

Case 1 —*Glioma of the right frontal lobe Reflex grasping with left hand Exploratory operation followed by death Necropsy*

B , a man, aged 39, a private in the Marine Corps, was admitted to the U S Naval Hospital, Feb 6, 1928, because of headache vomiting and convulsions The illness began on *January 12* with a *nocturnal* convulsion noted by his wife Breathing was stertorous and the left arm was extended straight from the shoulder for about ten minutes, later becoming affected with a coarse tremor lasting twenty minutes The patient did not have any knowledge of the convulsion after regaining consciousness but manifested weakness in the left leg and in both arms the following morning Headache began immediately following this episode and progressed rapidly to great severity

January 24, he drove his car over an embankment, possibly during a petit mal attack, *although he said that he did not lose consciousness* at any time The headache increased, somnolence developed, and projectile vomiting occurred

Examination on admission to the hospital disclosed marked clouding of consciousness, and lethargy The patient was oriented, did not express any hallucinations or delusions, and showed good intelligence In standing with his eyes closed he fell backward and to the left Power was slightly less in the left hand The cranial nerves were *normal and the pupils* equal, reacting rather sluggishly to light the eyegrounds did not present choked disk or retinitis The tendon reflexes were normal, the cremasteric reflexes weak, and the abdominal reflexes absent on both sides There were no sensory disturbances The spinal fluid Kahn reaction was negative, the cell count 6 per cubic millimeter there was a slight trace of globulin, and the colloidal gold curve was negative The roentgenogram of the skull was negative.

During the week *following admission the left arm and leg* became useless and characteristic reflex grasping developed in the left hand Whenever an object was brought into the palm *it was tightly grasped and the patient was unable to let go of* it even though he attempted to do so Observation of the extensor tendons left no doubt of the voluntary efforts of the patient to release the object yet it was many seconds before to the disease when no organic basis can be found for it,[8] while "symptomatic narcolepsy" is the term applied to the disease when it rests on some organic disturbance in the brain (arteriosclerosis, diabetes, cerebral tumor or encephalitis) Many of the cases of so called symptomatic narcolepsy described do not show both sleeping and loss of tone attacks, therefore, many of them should not be included under this term

REPORT OF CASE

The following is a case of true symptomatic narcolepsy following an attack of acute epidemic encephalitis

Miss A F, a school teacher, aged 36, with negative family and past histories, was first seen by me in consultation with Dr J H Wallis of Rice Lake, Wis, in August, 1928 In 1920 she had an attack of "influenza" lasting for about five weeks, during which time she had headache and some fever, and was unusually drowsy during the day but could not sleep at night She recovered from the acute attack—which in all probability was acute epidemic encephalitis—but since then has been troubled with diurnal attacks of involuntary sleep and periodic loss of muscular tone resulting from emotional stimulus Part of her written account of the illness reads as follows

"No excitement or commotion has the power to overcome that sleep I often sleep as often as five or six times a day This is not a natural sleep but a dead sleep which comes without warning Many times I realize that people are speaking to me and I understand what they are saying but am powerless to answer At times I can be awakened instantly by the sound of the voice, but when I am awakened memory fails me, and I am humiliated by being forced to admit my inability to remember what the conversation has been The longest sleeps generally come at about 10, 2 and 6 o'clock, sometimes lasting as long as two hours

"I know that if I wish to go to an entertainment of any kind I cannot trust myself to laugh If I must laugh or be where there is much excitement I must place my hands on some object that will hold my weight. My strength leaves me until I haven't the strength of a tiny babe At first my knees double up and I fall From there the weakness spreads to my neck and my head falls forward or sideways I have no control of any of my muscles the weakness passing through my shoulders and arms until it spreads over my whole body If I have anything in my hands I drop it. This loss of strength and control passes through my whole system like a tornado playing havoc wherever it goes—all due to a smile or a little excitement The strength soon returns and I have control of the muscles again"

In addition to these symptoms the patient complained of muscular stiffness tremor of the hands almost constant headache, extreme irritability restlessness and insomnia.

Neurologic examination revealed a moderate hypertonus of all the muscles, the right pupil slightly larger than the left, slight weakness of the right facial nerve, a fine tremor of both extended hands some dysmetria of the right arm, and exaggeration of all deep tendon reflexes Blood pressure, urinalysis and blood studies gave normal observations The blood Wassermann reaction was negative

Since I first saw her the patient has had about every conceivable form of treatment without avail Diet internal glandular therapy, sodium salicylate, belladonna stramonium, acriflavine and various sedatives all have failed to give relief It is a remarkable testimonial to the patient s grit and perseverance that in spite of her handicap she has continued to teach school without loss of time

COMMENT

This case is undoubtedly one of symptomatic narcolepsy following epidemic encephalitis That lesions in the midbrain can cause sleep has been shown by Hess[9] who injected ergotamine into the third ventricle of animals and produced periodic sleep Also Pette[10] saw abnormal sleep resulting from a circumscribed lesion of the central gray substance in the third ventricle Further Adler[11] describes a case of sleep disturbance [illegible] an abscess the size of a pea in the region of the third ventricle Straussler[12] has observed similar phenomena in lesions of the infundibulum In the light of these reports, and since the pathologic changes in chronic encephalitis involve principally the midbrain, the pathologic connection between symptomatic narcolepsy and chronic encephalitis is established

CONCLUSIONS

1 Narcolepsy as first described by Gelineau and Redlich has two symptoms (*a*) periodic attacks of involuntary sleep, and (*b*) attacks of temporary and complete loss of tone in the skeletal musculature following an emotional stimulus

2 In "essential" narcolepsy, no organic basis for the syndrome can be found "Symptomatic" narcolepsy is the result of some organic lesion in the brain

3 Reports of sleep disturbances following lesions in the third ventricle and the fact that in chronic encephalitis the pathologic changes are principally in the midbrain establish the relationship between chronic encephalitis and narcolepsy

4 In the present case of narcolepsy following encephalitis, all forms of treatment have failed

1125 Lowry Medical Arts Building

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12 Straussler Klin Wchnschr 1927 number 18 p 878

CAROTINEMIA IN AN ADULT

W J Potts MD, Oak Park Ill.

Carotinemia as a clinical entity was established by the work of Hess and Meyers[1] in 1923 They observed a peculiar symptomless discoloration of the cheeks, alae nasi and forehead, and occasionally a generalized tinting of the skin in children fed on a high carrot diet They were able to produce the pigmentation by the administration of carotin, and they saw it disappear when the pigment was withheld and the pigment-bearing vegetables were eliminated from the diet. It was also discovered that spinach, oranges and egg yolk contain a large amount of carotin

DeBuys[2] reported two interesting cases of carotinemia in new-born children, observed and studied in 1911 and 1913, and diagnosed in 1923 after the publication of the paper by Hess and Meyers A high vegetable diet had been advised for the mother, who took the advice too literally and practically lived on green vegetables Each child when born was normal in every way but markedly pigmented The mother's milk was colored a deep orange-yellow When the children were removed from the breast and put on artificial feeding, the discoloration promptly faded The mother returned to a balanced diet and consequently to normal color

Xanthosis diabetica, a yellow discoloration of the skin occurring in diabetic patients on a high vegetable diet, was reported by von Noorden in 1904 It has since been described by Stoeltzner[3] as pseudo-icterus The work of Palmer and his associates on carotin has cleared up the cause of these pigmentations and put them all in the class of carotinemia. They have demonstrated that the color of body fat, milk, egg yolk, and serum in cases such as the ones here described is due to carotin which in no way differs from the pigment isolated from vegetables

REPORT OF CASE

History—Mrs Mary B, aged 48, entered the Presbyterian Hospital because of a slowly deepening jaundice, pain in the epigastrium and constipation Six months previous to entry she had spent six weeks at a sanatorium under treatment for these symptoms Following a fast of forty five days' she was put on a diet limited strictly to vegetables and fruits, and dismissed After approximately three months of this regimen she noticed that her skin, especially the palms of her hands, the soles of her feet, and her face, was becoming yellow This discoloration had deepened to a rather marked general orange yellow at the time of presentation The pain in the epigastrium and the constipation had been present for six years, and were typical of a mucous colitis brought on by the prolonged injudicious use of cathartics the grip relaxed It was increased by attempts to withdraw the object but could be abolished by asking the patient to perform some common act with his right hand, in other words, by distracting his attention At times, when the object was caught merely by the flexed finger tips the reflex became clonic in type, although there was no wrist clonus or marked exaggeration of tendon reflexes As a rule the left arm of the patient rested at his side and he seemed to make no effort to move it In sitting up the arm hung at his side neglected Yet in voluntary movements it was not greatly inferior to the opposite limb in muscular power, although fine movements were practically abolished by the marked tendency toward tonic innervation The tendon reflexes were somewhat increased in the left arm, Mayer s sign was exaggerated and Hoffman s sign was slightly positive There was no thumb sign The sense of position was normal in the fingers Tests for apraxia were unsatisfactory owing to the obnubilation of the patient and the poor coordination of the left hand He was able to perform acts of buttoning, writing, and striking a match with the right hand The left leg was considerably weaker than the right and showed exaggerated reflexes and abortive ankle clonus but no Babinski sign Papilledema had appeared by this time, being more marked on the right side A diagnosis of rapidly growing glioma of the right frontal lobe was made and the patient was submitted to exploratory operation by Dr H H Kerr Preliminary ventriculography disclosed marked depression of both anterior horns with partial obliteration of that on the right side On exposure of the dura there was great pressure, which was relieved by tapping a cavity in the growth and removing 25 cc of brownish red fluid Zenker's solution was injected through the cannula and the flap closed with decompression The patient failed to regain consciousness and died about twelve hours later

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[1] Hess A F and Meyers V C Carotinemia A New Clinical Picture J A M A 73 1743 (Dec 6) 1919

[2] DeBuys L R Tr Mississippi State Medical Association, 1[illegible] pp 133 138

[3] Stoeltzner W Munchen med Wchnschr 66: 419 1919

Necropsy disclosed a soft tumor, cystic and hemorrhagic, lying in the right frontal lobe appearing on the medial surface over the genu of the corpus callosum (fig 1) It measured 62 by 46 by 60 mm and contained a cavity the walls of which were fixed by the fluid injected The gross and microscopic picture corresponded with that of spongioblastoma multiforme

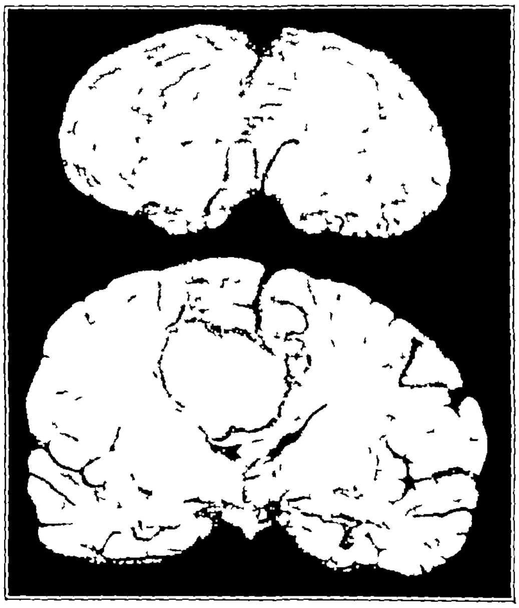

Fig 2 (case 2) —Tuberculoma of left frontal lobe with implantation in the right frontal lobe another mass in the right cerebellar hemisphere The patient showed reflex grasping on both sides more marked on the right.

Case 2—*Tubercles on each side of median longitudinal fissure Bilateral reflex grasping Death from generalized tuberculosis*

J L, a man aged 35, colored, was admitted to Gallinger Hospital Dec 18, 1927, suffering from delusions of persecution and alcoholism The history was very fragmentary and unsatisfactory For more than a year he had suffered from severe headaches and dizzy spells with staggering Severe epistaxis had occurred two or three times On one occasion his jaws remained locked for twenty-four hours At Gallinger Hospital he had one convulsion He said that he felt well except for headache, but he had lost weight and suffered from coughing spells during the three months preceding admission He continued working until he suddenly became blind in October 1927, supposedly as a result of wood alcohol poisoning He was quite demented on admission to St Elizabeth s Hospital and had to be helped about the ward He had an evening rise in temperature and presented advanced miliary tuberculosis of the lungs

On neurologic examination Feb 29 1928 the patient appeared weak poorly nourished uncooperative and listless He said he was able to see on Tuesdays but at no other time He could walk about the ward avoiding objects and his blindness was believed to be hysterical The pupils were equal and did not react to light The eyegrounds showed sharp outlines of the optic disks without atrophy or swelling but with some overfilling of the veins There was no paralysis although the patient tended to fall backward when standing The tendon reflexes in both upper and lower limbs were quite active and equal Examination of the sensibility could not be carried out The neck was stiff, with a suggestive Brudzinski sign and a positive Kernig sign on each side. Vasomotor and pilomotor phenomena were unusually active. The abdominal reflexes were brisk and equal, the cremasteric reflex could not be obtained on account of the tonic contraction of this muscle There was some fibrillary twitching in the quadriceps on each side

When the fingers were inserted into the right palm and the thenar eminence was rubbed, there was reflex grasping which persisted in spite of commands to the patient to let go Not infrequently the grasping was clonic It was increased by pulling outward on the flexed fingers, but the fingers could be pried loose and there was no groping after the retreating object On the left side there was a suggestion of grasping when the thenar eminence was rubbed and the object withdrawn from the palm

Examination of the spinal fluid March 5 revealed a pressure of 28 mm of mercury, 3 cells per cubic millimeter, and a trace of protein, there was a negative Wassermann reaction and a gold curve of 0023332100 At a second examination March 14, there were 19 cells per cubic millimeter the protein was 2 plus and the gold curve 0012233200 The disproportion between the clinical signs and the spinal fluid observations should have suggested the proper diagnosis, but the patient came to necropsy with the diagnosis of tuberculous meningitis Death occurred, March 23

At necropsy there was widespread tuberculosis, and a fibrocaseous mass weighing 75 Gm replacing the right suprarenal gland In the left cerebral hemisphere there was a fusiform tuberculoma measuring about 10 by 5 cm, lying subcortically close to the median fissure and breaking through the cortex into it about 3 cm behind the genu of the corpus callosum In the opposite hemisphere at this point there was apparently a secondary deposit centering about the callosomarginal fissure and involving little but the cortex (fig 2) Smaller tubercles were found in both occipital lobes not directly in the visual pathways one in the base of the pons on the left side and a conglomerate mass measuring 2 by 3 cm in the right cerebellar hemisphere

Case 3—*Arteriosclerotic dementia following repeated vascular insults Bilateral forced grasping and groping, disturbance of coordination in feeding Death from arteriosclerosis Atrophy of frontal lobes*

G K, a woman, aged 75, formerly a clerk in one of the federal departments was taken up by the police because she insisted on going to the office after she had been furloughed For several months preceding this she had been forgetful, inefficient and careless On admission to St Elizabeth s Hospital she was distractible, and she showed some fluctuation in mood being usually amiable, but sometimes irritable In 1923, a few months after her admission she had a cerebral accident, which caused confusion fever dyspnea tachycardia and transient unconsciousness She recovered in a few days becoming quieter and more demented A similar attack in 1926 left her bedridden mumbling spoonfed and untidy

When examined April 15 1928 she was completely demented aphasic and bedridden The skin had a clear pallor the peripheral and retinal vessels showed well marked sclerosis, and there

Carnegie Institution's Silver Anniversary—The twenty-fifth anniversary of the beginning of active research by the Carnegie Institution of Washington was celebrated at Cold Spring Harbor, Long Island, May 31 Exhibitions were arranged to show what had been accomplished by the department of genetics and members of the board of trustees and the president presented addresses The public was invited to the exhibitions, June 1-2 Further celebration will take place in August in San Francisco arranged by the department of terrestial magnetism of the Carnegie Institution This will relate closely to the activities of the nonmagnetic ship *Carnegie* which, although out on a three-year cruise will be in San Francisco then The celebration will be held aboard the ship The initial work of the department of terrestial magnetism at sea was done in the Pacific Ocean by the brigantine *Galilee* which was outfitted in San Francisco (THE JOURNAL, July 14 1928 p 106)

Medical Bills in Congress.—S 1538 introduced by Senator Brookhart, Iowa proposes to amend the World War Veterans Act to provide compensation for disability or death of a World War veteran, even though the disability or death be not of service origin H Res 57, introduced by Representative Black New York proposes that the House of Representatives request the President to call an international conference to limit the production manufacture, sale and distribution of narcotic drugs to the legitimate medical and scientific requirements of the world. H R 4124, introduced by Representative McSwain South Carolina, proposes to authorize the Arlington Cemetery Commission to set apart a suitable plot of ground in Arlington National Cemetery for the burial of the bodies of the heroes of the fight against yellow fever and to erect an imposing monument to the memory of the entire group H R. 4130 introduced by Representative Bland Virginia proposes to authorize an appropriation of $630 000 for the construction of additional hospital facilities and physicians quarters at the Southern Branch of the National Home for Disabled Volunteer Soldiers at Hampton, Va

Honorary Degrees—The following honorary degrees were awarded by the colleges indicated at the recent commencements

Arthur H Compton Ph.D professor of physics University of Chicago D Sc. Yale.
William H Park, M D professor of bacteriology and hygiene New York University and Bellevue Medical College D Sc Yale
William J Mayo M.D Rochester Minn D Sc. Northwestern University
William H Carmalt M D emeritus professor of principles and practice of surgery Yale University School of Medicine D Sc Yale
Robert A Millikan Ph D Norman Bridge Laboratory of Physics California Institute of Technology LL D New York University
William H Wilmer M.D director Wilmer Ophthalmological Institute Johns Hopkins University Baltimore D Sc New York University
William T Bovie Ph.D professor of biophysics Northwestern University Medical School Chicago D Sc. Albion College
Chester H Sample M D Saginaw Mich LL D University of Detroit
Thomas F Abercrombie M.D state health commissioner of Georgia D P H University of Georgia
Frank Vinsonhaler M D dean University of Arkansas School of Medicine LL D University of Arkansas.
James A Harten M D New Haven Conn M Sc. University of Pennsylvania.
John F Barnhill M.D Indianapolis past president of the American Laryngological, Rhinological and Otological Society LL D Indiana University
Lafayette Page M D Indianapolis professor of otology laryngology and rhinology Indiana University School of Medicine LL.D Indiana University
William N Wishard M D Indianapolis professor of genito-urinary surgery Indiana University School of Medicine D Sc DePauw University

Society News—The International Society for Crippled Children has arranged a conference of physicians public officials and social workers at Geneva Switzerland July 28 August 2 The subjects for discussion include finding crippled children examination, diagnosis and hospitalization relations of practitioners, education of the crippled child and the prevention of causes of crippling conditions Mr Edgar F Allen Elyria Ohio president of the society, will open the conference with an address on 'Evolution of the Problem of the Crippled Child ——Delta Omega public health fraternity recently elected to honorary membership Surg Gen Hugh S Cumming U S Public Health Service Dr Frederick F Russell of the Rockefeller Foundation and President Hoover Dr Charles V Chapin for many years health officer of Providence R I is the only other honorary member of the society ——The American Association of Medical Milk Commissions and the Certified Milk Producers' Association of America held a joint meeting June 24-25 at Montreal Karl F Meyer Ph D professor of bacteriology and director of the Hooper Foundation University of California Medical School spoke on 'Undulant Fever in the Light of Recent Observations", W H Diffenbach, 'Milk Production and Facts in Regard to a New Test for the Detection of Tuberculosis ' and Prof William D Frost of the University of Wisconsin, Madison, 'Examination of Cows and Employees for Streptococcus Epidemicus Carriers' ——Dr Rene Sand, Paris was elected president of the first International Hospital Congress which met in Atlantic City June 14 and E H Lewinski Corwin Ph D, secretary-general

A Mine of Official Information.—The new edition of the American Medical Directory which has just been published by the American Medical Association contains in one volume a huge amount of information arranged in convenient form One feature is an abstract of the laws which govern medical practice in each state the names of members of licensing boards and state boards of health the names of city and county health officers the officers of state and county medical societies and of those physicians who are in the government services On page 49 are the names of pathologists who are conducting clinical laboratories which have been approved by the Council on Medical Education and Hospitals of the American Medical Association The directory is composed largely however, of authentic data about more than 168 000 physicians in the United States its possessions and Canada

CANADA

Annual Medical Meeting and Election.—The Canadian Medical Association held its annual meeting at Montreal from June 17 to 21 The program included guest speakers, clinics business sessions meetings of allied associations, and entertainment including a golf tournament on the Dixie links, the winner of which was awarded the Ontario cup' Among the guests were Prof G B Roatta, Florence Italy who spoke on 'Child Welfare the Basis of Public Health Sir St Clair Thomson London England Intrinsic Cancer of the Larynx Lasting Cure in 80 Per Cent of Cases by Laryngofissure" Prof E Rist, Paris Sudden Onset of Pulmonary Tuberculosis and Its Lobar Localizations" Dr F J H Campbell, London 'Mental Changes in Physical Disease Dr Charles F McKhann, Jr, Boston 'Early Diagnosis of Poliomyelitis' with moving pictures, Drs James A Doull Baltimore 'Progress in Studies in Acute Respiratory Diseases', P F Armand-DeLille, Paris 'Diagnosis of Early Stages of Pulmonary Tuberculosis in Infancy and Childhood L Ambard Strasbourg France, 'Significance of Hyperglycemia in Diabetes" and J A Cairns-Forsyth London England, "Surgery of the Biliary Tract and Pancreas' The Canadian Public Health Association the Womens Medical Association the French-Speaking Physicians Association, and the Canadian Social Hygiene Council met simultaneously The officers elected by the council of the medical association at this meeting were Drs Alfred T Bazin Montreal president William Harvey Smith Winnipeg president elect, Thomas C Routley Toronto general secretary, reelected and Frank S Patch Montreal honorary treasurer The annual banquet was attended by a distinguished company but there were no speeches The council passed a resolution regretting the resignation of Dr Alexander D Blackader as editor of the associations journal after ten years of service

Osler's Remains Rest Among His Books—The library which Sir William Osler bequeathed to McGill University Montreal was dedicated May 29 In the large audience were the sister of Lady Osler Mrs H B Chapin Boston the nephew of Sir William Mr H S Osler the prime minister of Canada and other distinguished persons Dr William Sydney Thayer Baltimore President of the American Medical Association long a colleague and close friend delivered an eloquent and beautiful appreciation of Osler as a man as a physician and as a writer Leonard L Mackall in the New York *Herald Tribune* writes that the books were not sent to Montreal until Bibliotheca Osleriana had been completed It was Oslers request that the catalogue which had been in preparation since before his death in 1919 in case of death be completed by Lady Osler and his friends Dr W W Francis who had lived in Oslers home for years Dr Thomas A Malloch librarian of the New York Academy of Medicine Mr H H Hill and Mr Mackall With the exception of Lady Osler who died last year they were present at the dedication in Montreal Osler as a young physician, spent more time and money than he could afford to collect scientific journals In 1884 he was called to Philadelphia where he continued to collect journals Many of his rare books were acquired while in Baltimore He was so was some contracture of the lower limbs The upper limbs were hypertonic but not contractured, and both of them at times were waved about apparently aimlessly, groping now for the bedclothes, now toward the face or the gown

The face preserved a smiling blank expression throughout the examination, except when painful pressure was exerted over bony areas She remained as nearly passive as any patient could. The head was normal to palpation. The pupils were of medium size, equal and regular, and they responded to light The patient resisted opening of the eyes The teeth were all missing and the maxilla was atrophic. When an attempt was made to open the mouth for purposes of examination it was firmly closed and only with considerable difficulty could a tongue depressor be introduced This closure was seemingly a reflex action, as was apparent during feeding The jaw jerk was very active and the labial muscles contracted sharply on percussion The neck was moderately stiff The thorax was normal, the abdomen thin-walled, with brisk cutaneous reflexes

The upper extremities were held flexed over the chest Both hands were usually made into a loose fist, the thumb fitting against the curve of the index The right hand was more active in grasping and groping, but the left also participated When the attempt was made to draw away the top sheet, the patient, after groping about a little, caught hold of the edge and held fast to it in spite of rather notable traction If the attempt was made to open her mouth, the right hand would be brought up in a groping manner, catch hold of the hand of the examiner, and hold fast If during this motion it was intercepted, the intercepting object would be grasped just as forcibly, and the left hand would be brought up slowly with some oscillations and slow tremors, to catch the hand that was pressing on her jaw Similarly, when an attempt was made to expose the chest, the hands were brought up and clutched the chemise tightly

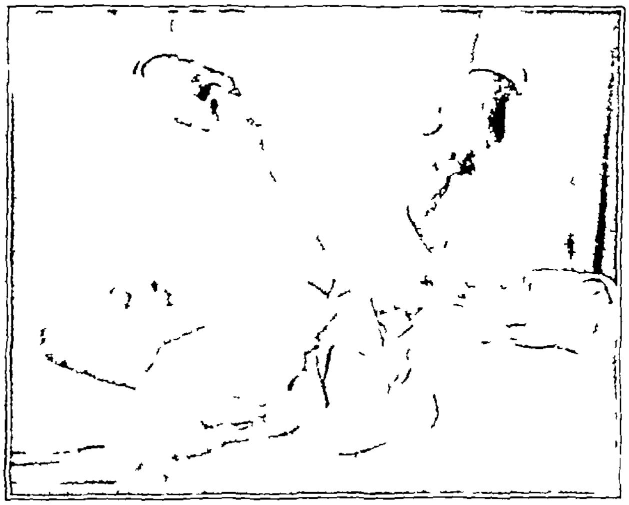

Fig. 3 (case 3)—Bilateral reflex grasping The patient's head and shoulders could be raised from the bed.

When the examiner's fingers were introduced into the patient's palm they were immediately grasped (fig 3) and the force of the grasp increased as attempts were made to withdraw the fingers or as the palm was scratched. The grasp continued for a minute at a time, even if no attempt at withdrawal was made During this period the hand was occasionally animated by slow rhythmic tremors about two to three a second, and of considerable amplitude, consisting mostly of flexion and extension of the wrist, although the fingers occasionally participated, the rate being about twice as fast The tremor would come and go without apparent cause When the hand lay passively on the chest, stroking along the thenar eminence resulted in flexion adduction and pronation with grasping movements of the fingers When the hypothenar eminence was stroked the patient's hand again groped toward the stimulating object At times the hand could be led back and forth across the bed by such maneuvering If at any time it caught the object, the grasp immediately tightened and remained fixed A painful stimulus upset the grasping mechanism somewhat When a pocket knife with all blades open was placed in her hand, it was grasped as any other object, but the grasp was light When the points were pressed against the skin, the hand opened As soon as the pressure was relaxed and the points ceased sticking the grasp was resumed. The patient did not fumble with the object or attempt to turn it about so that it would not prick her During all such operations the patient's face bore no trace of emotion She lay with her eyes half closed, apparently oblivious to the manipulations

The tendon and periosteal reflexes were exaggerated, without clonus Mayer's and Hoffmann's signs were exaggerated on the right side and present on the left. Scratching the hypothenar eminence resulted sometimes in adduction of the thumb, sometimes in flexion of the wrist with partial withdrawal of the hand The nutrition of the skin and muscles seemed quite good There was slight contracture The patient responded to pinpricks and deep pressure by withdrawal of the hand, but no other tests for sensibility could be carried out on account of the dementia

The lower limbs were slightly contracted in flexion The muscles were rather atrophic and hypertonic but under fairly good coordinated control The tendon reflexes were exaggerated, without clonus, and a negative Babinski sign was observed A light tap on the sole at the base of the second toe was followed by flexion of all the toes and adduction of the lesser ones At times the ankle was extended The reaction occurred with very little latency, was repeated time and again in a stereotyped manner, required a very slight stimulus, and lasted only a moment. Forcible stroking of the tibial crest yielded a more tonic response of the same sort These reactions were a little more pronounced on the right side than on the left but were identical in kind. The patient could not sit up or walk. Tests for sensibility in the lower extremities were limited to pricking and pinching, to both of which the patient responded by withdrawal of the foot She made no outcry or other objection

Feeding, according to the nurses' observations and our own, was a difficult and complicated affair The patient willingly partook of everything, without making any objections, she was not resistive in any way, yet she was practically unable to open her mouth for the purpose of taking food When she attempted to do so her mouth closed with a snap, and any attempt to open it by external force met with invincible resistance. If a tongue-blade was introduced into the mouth the patient made efforts to get rid of it, working against it with the tongue so that it was difficult to introduce it into the pharynx When finally it could be pressed against the posterior pharyngeal wall, it provoked a mild gag reflex with wide opening of the mouth

In feeding the patient very small morsels had to be given, otherwise they could not be introduced into the mouth Even the small ones were frequently rejected, the tongue curling over them and pushing them through the lips The patient could swallow readily and apparently enjoyed her food, but she could not manipulate it properly in her mouth We have observed this phenomenon in an infant, aged 8 months, who was just beginning to take solid food The patient's actions resembled so closely the behavior of the infant that she was given a nursing bottle and nipple. As soon as a drop of milk touched her lips she opened her mouth wide enough for the introduction of the nipple, rolled it about with her tongue, and sucked on it There was no rhythmic contraction of the jaws, although within a few minutes she had taken about 4 ounces of milk The nipple was easily released from the mouth. Moving it about in the mouth seemed to stimulate sucking She held the bottle in her hand for a time, but lowered it too much to take more milk She would pull it from her mouth, at the same time clamping the nipple till it was feared that she would pull the nipple off She seemed satisfied after she had taken 5 ounces and shut her mouth against more.

At necropsy the body weighed 34 5 Kg and measured 147 cm There were contractures of the lower limbs Except for atrophy and arteriosclerosis the organs did not show significant changes The brain weighed only 890 Gm and was surrounded by 200 cc. fluid The meninges were slightly thickened and showed enlarged arachnoid villi The vessels at the base were tortuous and calcified There was extreme atrophy of the hemispheres, especially in the frontal regions, where the fissures gaped widely and the convolutions were narrowed and firm. No focal lesions were visible externally The ventricles were widely dilated and extended much farther forward than usual, leaving a thin layer of white matter between the ependyma and the cortex This white matter was of good color except at the lateral margin of the ventricle, where it had a grayish translucent appearance In the right hemisphere there were two infarcts, one involving the anterior limb of the internal capsule with the adjacent gray nuclei the other destroying the inner portion of the globus pallidus The basal ganglions were markedly atrophic, but no other focal lesions were evident. The cerebellum and brain stem were likewise atrophic.

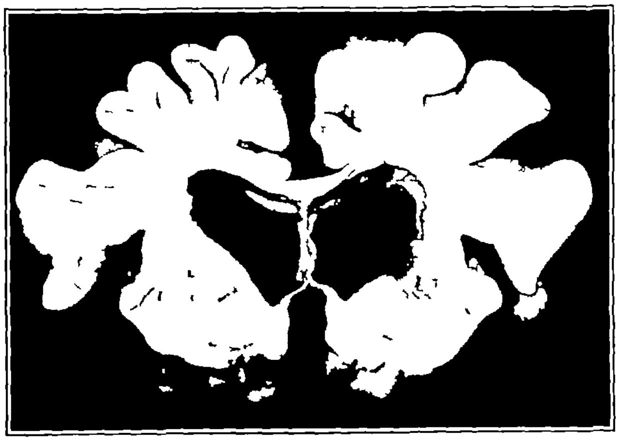
Fig 4 (case 3) —Atrophy of both hemispheres which was especially marked in the frontal regions and dilatation of the lateral ventricles. The patient showed bilateral reflex grasping

The phenomena in this case were attributed to the extreme atrophy of the frontal lobes rather than to the focal lesions encountered in the right hemisphere

Case 4—*Cerebral arteriosclerosis with dementia bilateral reflex grasping death from cardiac failure Atrophy of both frontal lobes and pons*

B M, a woman, aged 60, was admitted to St Elizabeth's Hospital, Nov 1, 1927, and died Aug 7, 1928 Her past history was unimportant, her father had died at 60 of a paralytic stroke Her illness began with a fall in September, 1926, after which she was weak on the right side and could not speak for several hours On admission she showed residuals of right hemiplegia and loss of memory, she was completely disoriented and was untidy in her habits Within a few months she became completely demented and mute, and early in August, 1928, developed fever and signs of pulmonary consolidation Examination on July 23, two weeks before death, when she was still in her usual condition, revealed the following The head was normal, and the pupils were equal and active, the eyegrounds were not satisfactorily observed because of the constant motion The patient resisted opening of the eyes, apparently a manifestation of "tonic innervation" The face was calm and expressionless during the examination, and on supra-orbital pressure grimaced normally, no weakness being detected. The tongue was protruded with slight tremor When an attempt was made to open the mouth by pressing on the chin there was tonic closure of the mouth, and when the tongue blade was introduced it provoked puckering of the mouth and coordinated resistance by the lips, jaw and tongue, yet to judge from her facial expression, the patient did not seem to resent the maneuver According to the nurses she was able to take only very small morsels of food, any large piece being rejected as in case 3 To all appearances she relished her food and could chew it without difficulty and swallow normally A mild gag reflex was elicited by pressure on the pharynx The hands were kept folded over the chest most of the time There was no atrophy or deformity, the muscles were weak but hypertonic with exaggerated tendon reflexes Hoffmann's and Mayer's signs were present on both sides, and Marie's sign on the right On stimulation of the palm and thenar eminence by deep stroking with the finger there was moderately active grasping on the right side, which quickly relaxed when the friction was discontinued There was some tremor present. On the left side the grasp was maintained, and there was some tendency to grope toward the covers, the chemise or the face. The grasp often became clonic in character and the hand was occasionally activated by slow rhythmic movements at the wrist The abdominal cutaneous reflexes were absent, but the rectus tendon reflexes were exaggerated

The lower limbs were in slight flexion contracture with hyperactive reflexes and bilateral ankle clonus and Babinski sign On the left side, although almost any stimulus would provoke dorsal extension of the great toe, a mild touch at the base of the second toe would occasionally provoke flexion of all the toes

Necropsy one hour postmortem revealed generalized arteriosclerosis with emphysema and bronchiectasis The brain was small, weighing only 940 Gm The frontal lobes seemed especially atrophic with narrow convolutions and pronounced fissures, and there was an excess of cerebral fluid, measuring 240 cc The pons was exceptionally atrophic, both sides appearing sunken and narrow The meninges were slightly opaque over the frontal area, the blood vessels were markedly sclerosed, dilated and tortuous Section of the brain after fixation revealed dilatation of the ventricles and reduction of the subcortical white matter, with a thin corpus callosum (fig 5) In the left frontal pole underlying the gyrus rectus opposite the genu was a small cystic infarct Farther posterior, where the ventricles opened, the white matter of the frontal lobes presented a translucent grayish aspect and was markedly diminished in amount On the right side, opposite the head of the caudate nucleus, lying in the anterior limb of the internal capsule, was a small cystic infarct The atrophy of the white matter was apparent throughout both frontal lobes but in no other portion of the brain Gross section of the pons revealed prominent pontile nuclei with thin pyramidal tracts

Case 5—*Paranoia with senile deterioration Flexion contracture on right, reflex grasping on left Death from cardiac failure Bilateral atrophy of white matter of frontal lobes and of the pons*

S M, a white woman, was admitted to St Elizabeth's Hospital April 20, 1906 and died Aug 18, 1928, at the age of 82 Her delusions of persecution drove her to seek redress from the President, and she was committed to the hospital She was meddlesome and officious and occasionally disturbed, and her delusions of grandeur and persecution subsided only with advancing deterioration No definite apoplectic insults were noted, yet in 1926 she became helpless and developed progressive contracture of the right arm and leg with severe dementia Death resulted from arteriosclerosis and cardiac failure

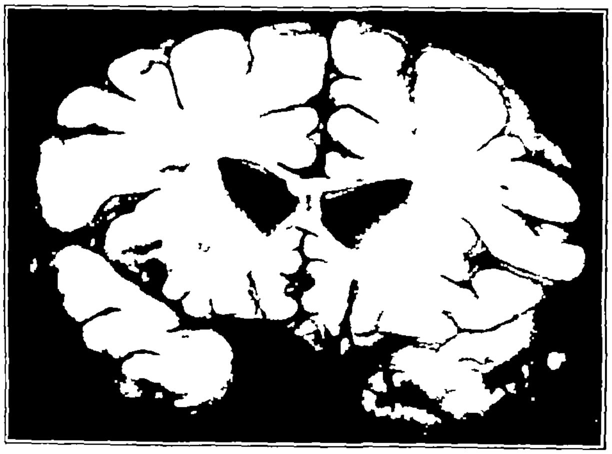
Fig 5 (case 4) —Atrophy and infarcts in both frontal lobes. Bilateral reflex grasping had been present.

At examination, July 23, 1928, the patient was practically deaf blind and mute. The pupils were small and equal, responding slightly to light. Cataracts prevented examination of the fundus. The eyeballs moved in conjunction without nystagmus. Facial mimicry was impoverished, although grimacing due to pain was symmetrical. There was little resistance to the introduction of a tongue blade, although the patient pursed up her lips. The mouth could be opened without difficulty by pressure on the chin. The pharyngeal reflex was weak.

The right arm was in strong contracture, the atrophic hand being folded on the chest with the index finger extended. The left upper limb was moved about freely by the patient. Hoffmann's sign was doubtfully present on each side, Mayer's sign was absent on the right, exaggerated on the left. Marie's sign was absent. Reflex grasping was elicited by forceful friction of the palm and thenar eminence on the left side but was liable to relax quickly unless traction was continued on the flexed fingers. There was no definite groping, although the left hand tremulously sought the site of painful disturbance in the lower limb. On both sides there was pronounced tremor, slow and rhythmic, brought out by action and quickly dying away. The left hand tended to grasp the bedclothes as they were drawn away but did not interfere with feeding. The tendon reflexes in both upper extremities were very active.

The abdominal reflexes were not obtained. The lower extremities were drawn up in flexion, which was invincible on the right. The Babinski sign was negative bilaterally, the Oppenheim negative on the right but positive on the left. The tendon reflexes were quite brisk but without clonus. Tapping at the base of the left second toe occasionally produced flexion of all the toes, but usually the whole foot was withdrawn with marked tremor.

The necropsy, performed forty-six hours after death, revealed senile emaciation (31.5 Kg.), and severe contracture of both legs and the right arm. Generalized arteriosclerosis with senile atrophy of all the organs was noted. The heart showed some fibrous changes in the myocardium. The brain was considerably shrunken, weighing only 923 Gm., and being surrounded by 230 cc. of fluid. The meninges were essentially normal, the arteries dilated, tortuous and atheromatous. No thromboses were encountered. The frontal lobes in particular were markedly atrophic, with narrow convolutions and broad fissures. The pons likewise showed general atrophy. Section after fixation revealed on both lateral aspects of the dilated anterior horns a grayish porous condition of the white matter but no definite areas of infarction. The two hemispheres seemed about equally affected. The cortex was thin, the corpus callosum not markedly atrophic. The basal ganglions were free from significant lesions. Section through the pons revealed reduction in the size of the ventral part, with prominent gray masses.

Delayed Operation in Tubal Disease.—Almost twenty years ago F. F. Simpson read before the American Gynecological Society a paper which revolutionized the treatment of tubal disease. Prior to that time its management had been purely surgical. Operation was done routinely as soon as the diagnosis was made and because it was done in the acute stage, ablation of the entire genital tract was often a necessary consequence. The result was not only a group of thoroughly unsexed women at least in the physiologic sense, but also a death rate approaching 20 per cent. Then came Simpson with his advocacy of the delayed operation with the plea that surgery should never be resorted to in acute salpingitis until the temperature had been consistently normal for at least three weeks even after repeated bimanual examination until there had been absolute recovery from the acute attack, and until there had been a complete absorption of the inflammatory exudate surrounding the primary focus of infection. These astounding proposals could not be ignored, revolutionary as they were because their advocate was able to show for them in a series of more than 400 cases the hitherto unheard of mortality of 1 per cent.—Miller, C. J.: Salpingitis: The Case for Expectant Treatment, *Am. J. Obst. & Gynec.* December 1928.

INFANTILE KIDNEY

ABRAHAM G. FLEISCHMAN, M.D.
AND
BOYD ANDERSON, M.D.
DES MOINES, IOWA

Numerous articles have appeared in medical journals dealing with kidney anomalies which are no longer considered rarities. When one investigates carefully the literature concerning infantile kidney, one is impressed with the scarcity of contributions.

Geraghty and Plaggemeyer[1] have discussed this unusual anomaly and have emphasized its infrequency. Doria[2] has reported a case discovered at necropsy. Robinson[3] covers the subject of atrophic or rudimentary kidney, he does not limit his discussion solely to the small kidney but considers all forms of atrophic kidney resulting from various diseases. Kaufman[4] described in detail a small kidney of infantile character, discovered at the time of an operation. Krotoszyner[5] gave his observation of anomalies of the urinary tract and reported a case of renal hypoplasia. Lewis and Bethune[6] reported a case of infantile kidney, complicated by an occlusion of the ureter with nephrolithiasis on the opposite side.

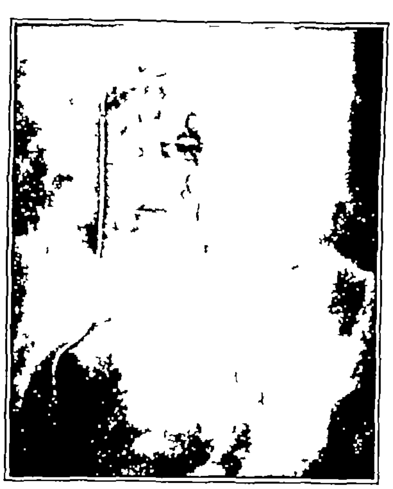
Fig. 1.—Pyelogram of the left kidney showing a distinct filling defect deformity.

Infantile kidney, often designated as congenital atrophic kidney or renal hypoplasia, is vitally important in urologic differential diagnosis and must be thought of whenever surgery of the other kidney is contemplated.

Our interest was aroused in the study of this relatively rare anomaly by an unusual case. Many theories have been offered as to the origin of this anomaly from the embryologic point of view, and all agree that it is the result of an arrested fetal development.

Sufficient evidence has been obtained by competent and well qualified investigators to substantiate the view that atrophic changes in a kidney can be the result of various diseases. It is extremely difficult, however, to differentiate between the small kidney that is the result of some acquired pathologic process and the kidney that is atrophic because of some congenital nondevelopment.

The size of these kidneys may vary from that of a small bean to that of an English walnut. The kidney is located usually in the same region as is the normal organ The pelvis of such a kidney may be precisely similar to the normal pelvis, with the exception of its distinct diminution in size, and arising from it there may be only a single calix or multiple calices which are also of minute dimensions Histologically it does not differ in any way from the usual normal kidney except for a noticeable decrease in its cortical area and a *marked deficiency in the number of glomeruli* The ureter of infantile kidney varies, it may be normal with some diminution in its size, or it may present other abnormalities

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[1] Geraghty, J. T., and Plaggemeyer, H. W.: The Practical Importance of Infantile Kidney in Renal Diagnosis, J. A. M. A. **61**: 2224 (Dec. 20) 1915.

[2] Doria, J. R. da C.: A Case of Atrophia Congenita of the Left Rim, Legal Medical Service, Bahia, 1924.

[3] Robinson, Bryon: Atrophic or Rudimentary Kidney, A. Practitioner News **38**: 535-560 (Sept.) 1912.

[4] Kaufman, L. R.: Congenital Atrophy of the Kidney, Urol. & Cutan. Rev. **26**: 283-286 (May) 1922.

[5] Krotoszyner, Martin: Observation on Anomalies of the Urinary Tract, Ann. Surg. **65**: 560 (May) 1917.

[6] Lewis, J. S., and Bethune, C. W.: Infantile Kidney and Occlusion of Ureter with Nephrolithiasis on the Opposite Side, Am. J. Urol. **8**: 599, 1912.

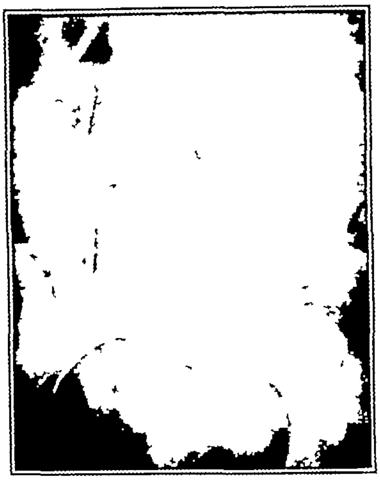

Fig 2 —Pyelogram of right infantile kidney showing rudimentary changes in all the calices particularly the minor ones

The clinical diagnosis of infantile kidney is not simple Frequently there are no symptoms that designate with any degree of certainty whether or not this pathologic entity is present The roentgenogram is seldom of assistance because of the extreme difficulty in obtaining proper outline in kidneys that show great diminution in size Cystoscopy, in conjunction with pyelography and the usual standard functional test, is of practical assistance

The bladder itself usually does not present anything of interest, unless some other concomitant pathologic condition is present Observation of the ureteral orifices reveals, in most instances, nothing abnormal Some investigators, however, describe atrophic changes of the circular muscle about the meatus

The ureteral catheter provides an excellent means of disclosing any obstruction that may be present along the ureter in its course from the bladder orifice to the renal pelvis, thereby determining permeability of the ureter on the side on which the infantile kidney exists

The examination of urine obtained from a ureteral catheterized specimen from the infantile kidney may not show anything abnormal with the usual chemical and microscopic tests, unless it is the seat of some pathologic process For this reason one must not take for granted that he is contending with a normal kidney , disastrous results may occur when dependence is placed on this observation Considerable decrease in the amount of urine will usually be observed Nevertheless too much significance cannot be attached to this, because on numerous occasions such diminution can be accounted for by a reflex inhibition following cystoscopic manipulation On the other hand, the other kidney will secrete proportionately greater quantities of urine, signifying a compensatory effort on its part

Renal functional tests are of great diagnostic help The dye output from an infantile kidney is always much diminished in amount while the output from the opposite kidney is increased The appearance of the dye from such a kidney is in most instances delayed while from the opposite kidney it is usually within the normal time limit An outstanding fact first recognized by Geraghty and Plaggemeyer,[1] pertaining to infantile kidneys was their ability to concentrate urea within normal limits, although not capable of performing the function of a full kidney

Pyelography also serves as a means through which a diagnosis of this condition can be determined, however, it is sometimes of doubtful value since the size of the kidney pelvis is usually not a reliable index of *the size of the kidney Examination of pyelograms will* reveal that the renal pelvis and calices of the kidney are exceedingly diminished in size in comparison with the normal In some cases the pelvic outline will be completely effaced, so that no true pelvis will be discernible In other instances the pelvis will assume a normal shape with an average size but the calices will be more or less obliterated The kidney may, however be so undeveloped that the pyelogram will not offer anything of importance by which such a kidney can be recognized

It is difficult at times to differentiate these forms of anomalies from the pyelographic point of view with other pathologic conditions of the kidney that present pyelograms similar to those of infantile kidney We refer particularly to renal tuberculosis, tumors of the kidney and those kidneys in which chronic inflammatory lesions predominate

REPORT OF CASE

History—Mrs W I, aged 53, an Iowan, first observed July 12, 1927, complained chiefly of pain under the left shoulder She had two children, aged 23 and 26 years The climacteric occurred at the age of 47 Her past medical history was negative with the exception of hyperacidity fifteen years previously and cystitis ten years previously

Jan 7, 1927, following a banquet in the evening, the patient was seized with a sudden acute pain under the left scapula which persisted for about six hours at its original site, radiating down the left side to about the crest of the ilium An hour after the onset she vomited gastric contents and continued to do so at frequent intervals for the next two or three hours She was given one-half grain (32 mg) of morphine but relief was not obtained, and half an hour later one-fourth grain (16 mg) of morphine was given There were no urinary symptoms and all specimens of urine were normal After she remained in bed for two weeks the pain gradually subsided, leaving a constant soreness July 9, she suffered a similar attack except that the condition came on more quickly than the previous one and the radiation of the pain at this time was directly across the upper part of the abdomen to the right costal margin The urine specimen at this time according to the family physician contained pus albumin, and a few red blood cells

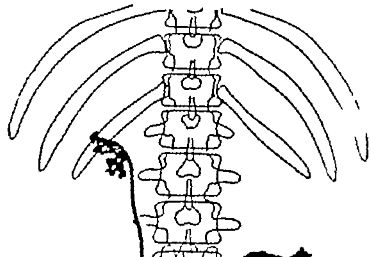

Fig 3 —Diagrammatic drawing made from the pyelograms to illustrate the difference between the two kidneys

July 12 the patient was rather pale and showed some evidence of loss of weight There was a general fulness of the left upper quadrant of the abdomen which moved slightly with respiration Palpation in this region revealed a firm rounded mass extending about three fingerbreadths below on the left costal margin It was somewhat tender anteriorly as one made pressure over the abdomen and left renal region Glandular enlargement was not present The blood pressure was 126 systolic and 80 diastolic, the pulse rate and the temperature were normal

The patient was advised to enter a hospital for a urologic examination

July 12, the blood count revealed red blood cells, 5,000,000, white blood cells, 18,600, polymorphonuclears, 89 per cent, lymphocytes, 10 per cent, transitionals, 1 per cent The urine specimen was straw colored, cloudy and acid, with a specific gravity of 1 020, there was a trace of albumin, but no sugar Microscopically, 2 or 3 white blood cells were found The blood urea was 6.5 mg per hundred cubic centimeters Phenolsulphonphthalein determination gave for the first specimen, 170 cc., 20 per cent, for the second specimen, 60 cc., 20 per cent, making a total for the two specimens of 40 per cent.

The first cystoscopic examination was done July 12 The bladder was essentially normal Ureteral catheters passed without any obstruction to each kidney

Phenolsulphonphthalein, when given intravenously, appeared on the left side in seven minutes the amount secreted was 20 per cent on the right side a very faint trace appeared in nineteen minutes The amount secreted could not be estimated because of the small quantity There was a scant amount of urine from the right kidney Microscopic examination showed an occasional leukocyte There was a moderate amount of urine from the left kidney, and an occasional leukocyte was revealed microscopically Sodium iodide 25 cc, was injected into the pelvis of the left kidney A pyelogram revealed a well defined filling defect of the upper pole and a flattening out of its pelvis, significant of renal neoplasm.

July 19, a second cystoscopic examination was done. The bladder was essentially normal Phenolsulphonphthalein was given intravenously it appeared after four and one-half minutes on the left side, and the amount secreted was 7.5 per cent on the right side a slight trace appeared in fifteen minutes the amount could not be estimated because of the small quantity secreted

There was a scant amount of urine from the right kidney There was a moderate amount of urine from the left kidney, microscopically both specimens revealed an occasional leukocyte, 4.5 cc of 12 5 per cent sodium iodide was injected into the pelvis of the right kidney The pyelogram revealed changes in both major and minor calices which indicated a rudimentary appearance

The third cystoscopic examination was done November 12 The bladder was essentially normal Indigo carmine was administered intravenously it appeared very strong from the left ureter in five minutes while only a faint trace came from the right in thirty minutes There was a scant amount of urine from the right kidney with an occasional leukocyte and 1 per cent urea nitrogen There was a moderate amount of urine from the left kidney, with an occasional leukocyte, and 1 per cent urea nitrogen.

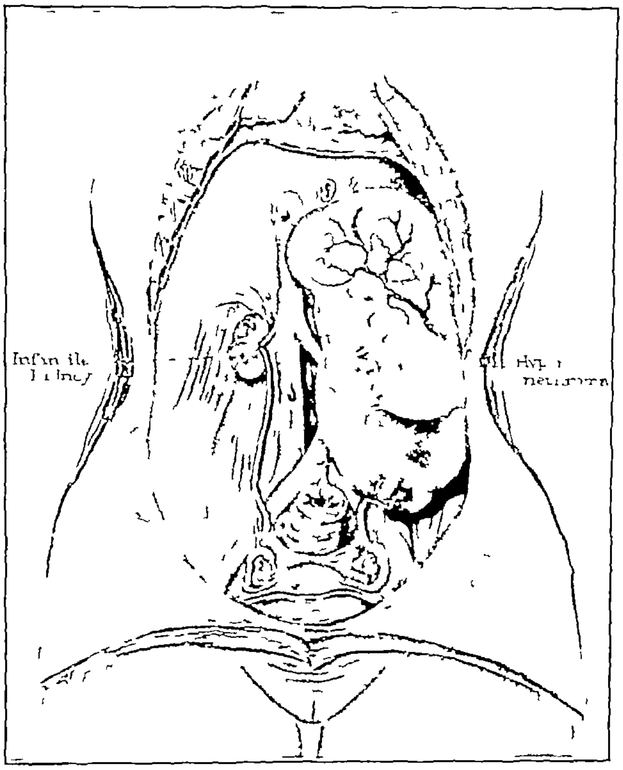

Fig 4 —Drawing made from necropsy observations showing the small infantile kidney on the right and an extensive hypernephroma of the left kidney

While the pyelogram of the right kidney was not typical in every degree in this instance of an infantile kidney, still there was some resemblance Other urologic observations were complete enough to make the diagnosis, namely, the normal character of the urine, the small amount of urine excreted, the normal urea concentration of the urine, the marked decrease in function as determined repeatedly by the various dye tests, and lastly, the normal function of the left kidney, although distinctly diseased

Operation and Result—Although the patient was informed on previous occasions that because of the marked functional incapacity of the right kidney operative procedure was not deemed advisable, she returned to the hospital, November 11, asking that surgical procedure be instituted November 17, an exploratory operation was performed with the idea that perhaps a partial resection of the left kidney could be carried out The attempt proved futile The patient became shocked to such an extent that further work had to be abandoned Postoperative measures were resorted to but the patient succumbed a few hours later During the operative exploration a massive tumor was observed occupying the greater portion of the left section of the abdomen Permission for a necropsy was requested and granted with the understanding that the structures involved should remain undisturbed

Necropsy—The left kidney was enormously enlarged It showed evidences that there had been a marked hypertrophy during life. Approximately the superior two thirds of the kidney was well encapsulated This was partially filled by a necrotic blood-tinged material It had the appearance of a degenerative hypernephroma The tumor mass and the remaining kidney substance was firmly fixed to the posterior parietes, extending mesially as far as the spinal column The remaining portion of the kidney measured 17 by 7 by 5 cm The lower pole contained a cyst about the size of an English walnut. The pelvis of the kidney had been entirely taken up by the tumor mass with the exception of the portion of the lower segment.

The right kidney on examination measured 6 by 3 by 2 cm It had the appearance of being normal in every respect but minute in size, and in comparison with its pyelogram was found to be much smaller The ureter entered the midportion of the kidney substance without a demonstrable formation of a pelvis and the examination of it did not disclose anything abnormal

CONCLUSIONS

The following are the important and salient facts that should be remembered with reference to infantile kidney

1 The function of an infantile kidney shows marked impairment as determined by the colorimetric dyes but still is able to excrete normal urine and concentrate urea in normal quantities

2 The function of a kidney in which disease is present is always greater than that of the supposedly normal infantile kidney

3 Pyelography is often of assistance in the diagnosis of this rare condition but cannot always be relied on

4 The decision before advising surgery in a diseased kidney when the other is infantile must be made cautiously because of the inability of the infantile kidney to undergo compensatory hypertrophy, an essential factor if a successful outcome is to be attained

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FAMILIAL PURPURA HEMORRHAGICA WITHOUT THROMBOPENIA

HEREDITARY HEMORRHAGIC THROMBASTHENIA*

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LOS ANGELES

"It is becoming increasingly evident that the group termed 'the hemorrhagic diseases' includes a large number of abnormal conditions, and that, at the present time, it is a fruitless task to attempt to unravel the various entities embraced by the clinical conditions which are assembled under this general head" This statement, made thirteen years ago in an oft quoted address by Alfred Hess,[1] is unfortunately still applicable today Nevertheless, as time goes on, the accumulation of a sufficient number of atypical cases with similar manifestations permits the formation of new groups, and subsequently necessitates a partial reclassification

Such a group of cases has been reported by Glanzmann[2] He has described in detail eight families with an unusual form of purpura and has proposed the name hereditary hemorrhagic thrombasthenia The disease affects both sexes and is usually transmitted by the females Briefly, the symptomatology consists of attacks of severe epistaxis and a tendency to develop ecchymotic areas spontaneously or following slight trauma The hemoglobin determinations and the red and white blood cell and differential counts showed only such a deviation from the normal as one would expect in an anemia due to loss of blood It was consistently noted that in nearly every case the number of platelets was normal or slightly increased The bleeding time was rarely prolonged, but apparently more blood appeared and a larger amount could be expressed from a minute finger wound than in normal controls The coagulation time was not altered, although occasionally the clot did not entirely solidify A failure of the clot to retract normally is a point particularly stressed by Glanzmann In many of the cases retraction was entirely absent, while in others it was only partly complete with occasional settling of the red cells to the bottom of the tube

Minot[3] has recently reported two families with a somewhat similar hemorrhagic condition In his cases the bleeding time was greatly prolonged and varied between thirty minutes and an hour or more The number of platelets, coagulation time, clot retraction and tourniquet test were normal The repeated attacks of epistaxis were without doubt a purpuric manifestation, as nasal telangiectases were not present Herniotomy, appendectomy and other operative procedures were not followed by any undue bleeding

Some cases that closely correspond to these, with prolongation of both the coagulation and the bleeding time, are recorded by Buckman[4] The number of platelets and the degree of clot retraction were not altered A family history of bleeding was lacking in one of his cases, but in all other respects it might well be included in this group

Giffin[5] has described a woman, aged 33, with epistaxis and other purpuric manifestations, designating the condition "severe hemorrhagic disease occurring in four generations" The bleeding time and prothrombin time were prolonged, whereas the number of platelets and coagulation time were normal He also reported the case of a girl, aged 9 years, with similar changes in the blood but without a family history of bleeding The bleeding time was prolonged despite a normal platelet count The clot did not retract Bleeding from the nose and gums was extremely severe and uninfluenced by injections of calcium, thromboplastic substances and some sixteen transfusions Splenectomy was performed with no immediate response and no alteration in the condition of the blood After a two months' interval, gradual improvement occurred and the child was finally discharged in good condition The spleen did not show any other pathologic changes, except an increase in fibrous tissue This case is mentioned again by Kennedy,[6] who has noted that since the onset of menstruation the periods have been accompanied by an alarming profuse menorrhagia The patient also had a severe hemorrhage following the extraction of a tooth

Little and Ayres[7] have traced a family with similar manifestations through five generations and report two typical cases Both showed a prolonged bleeding time with a normal number of platelets and no abnormality in the coagulation time In one patient bleeding persisted despite the usual therapeutic measures and splenectomy was finally resorted to Death occurred nine hours after the operation

Two more cases in a mother and son are briefly mentioned by Rosenthal[8] and classified as chronic thrombasthenic purpura The platelets were normal and the bleeding time and coagulation time prolonged He also refers to a similarly affected family described by Van der Zande[9]

A report of considerable interest from an historical point of view was mentioned by Jonathan Hutchinson[10] in a lecture delivered about fifty years ago His description is as follows

> A school boy was sent to me by Mr Harrison of Braintree, on account of repeated and alarming attacks of epistaxis They had been so profuse and so frequently recurrent that he had lost much of his color and strength The point, however, which most caused anxiety was that his elder brother had died of it. In him the liability had commenced at 13½ had persisted for two years and finally after continuing for four days it ended in

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* From the Children's Hospital

[1] Hess A. F The Blood and the Blood Vessels in Hemophilia and Other Hemorrhagic Diseases Arch. Int Med **17**: 203 (Feb) 1916

[2] Glanzmann E. Hereditare hamorrhagische Thrombasthenie Jahrb f Kinderh **88** 1-42 113 141 1918

[3] Minot G R. A Familial Hemorrhagic Condition Associated with Prolongation of the Bleeding Time Am. J M Sc. **175** 301 (March) 1928

[4] Buckman T E Atypical Pathologic Hemorrhage in Early Life Am J M Sc. **175** 307 (March) 1928

[5] Giffin H Z Unusual Types of Hemorrhagic Disease Am J M Sc **175** 44 (Jan) 1928

[6] Kennedy R. L. J Diseases of Children Benefited by Splenectomy J A. M A. **91** 874 (Sept. 22) 1928

[7] Little W D and Ayres W W Hemorrhagic Disease J A. M A. **91** 1251 (Oct. 27) 1928

[8] Rosenthal N The Blood Picture in Purpura J Lab & Clin Med **13** 303 (Jan.) 1928

[9] Van der Zande, F Pseudohemophilia Nederlandsch. Tijdschr v Geneesk **1** 544 (Feb 10) 1923 cited by Rosenthal

[10] Hutchinson Jonathan The Pedigree of Disease New York, William Wood and Co 1885 pp 101 102

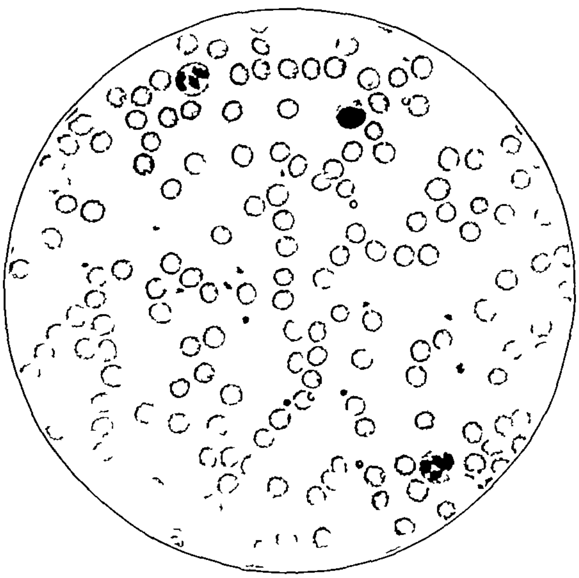
death Their paternal grandfather had suffered much from gout, and their father at the age of 22 had had severe epistaxis [And later he concludes with the following remark] My own experience as to retinitis hemorrhagica, blood patches in the conjunctiva, cerebral hemorrhage epistaxis and other allied disorders leads me however, to attach very great importance to the history of inherited gout as their frequent cause

REPORT OF CASE

We have had the opportunity to observe an additional case at the Children's Hospital in Los Angeles

A boy, aged 13, had been admitted to the wards on numerous occasions during the past nine years The mother had always *manifested a tendency toward easy bruising, and one brother* who died at 3 years of age from a street accident was similarly affected and also bled unduly from slight injuries The patient was first seen at the age of 3½ years with an infected scrotal hematoma resulting from a fall against a chair There was a history of frequent bruises and purpuric eruptions since birth. He had pertussis at 1 year and influenza during the epidemic of *1918* At *5* years of age *he had a severe nosebleed and had* since suffered from frequent recurrences At 6 years of age he had measles with a hemorrhage into the rash, and one month later was admitted with a generalized purpuric eruption.

Examination at this time showed large and inflamed tonsils enlarged cervical glands, dextrocardia, a soft systolic murmur best heard near the midline in the fourth right interspace, and a palpable liver edge on the left side. There was a moderate secondary anemia, normal white and differential counts, a bleeding time of six and one-half minutes, and a coagulation time of six minutes A diagnosis was made of situs transversus associated with an atypical blood dyscrasia



Blood smear with Wright s stain showing normal distribution of platelets × 400

He remained well after this attack until he was 7½ years old, at which time he had scarlet fever complicated by an acute nephritis As the nosebleeds became more frequent, a tonsillectomy and adenoidectomy were advised but permission could not be obtained *At the age of 10 years the patient* received two emergency transfusions because of prolonged nosebleeds He was given a transfusion again at 12 years and this time there were numerous ecchymotic areas over the entire body in addition to the epistaxis and oozing from the gums The blood pressure was 116 systolic and 72 diastolic The urine was normal, and *the Wassermann and tuberculin reactions were negative* Repeated nose and throat examinations did not show any evidence of localized bleeding points, ulcerations or telangiectases Bleeding finally stopped but recurred six weeks later He received four transfusions of about 150 cc each before improvement took place Roentgenograms of the long bones showed small but regular areas of lessened density in the lower metaphysis of the left femur and in the upper portion of the left tibia *The platelet count on this admission was never lower than* 200 000 per cubic millimeter of blood and on one occasion was as high as 322 500 The bleeding time varied from twenty to *thirty-five minutes* (Duke s method from ear) while the coagulation time remained between six and eight minutes Determinations of the blood calcium and phosphorus were normal The patient was seen at this time by Dr Harold K. Faber, who felt that he presented an unusual type of purpura After studying the fresh blood smears it was decided that splenectomy seemed inadvisable because of the presence of such a high proportion of *platelets* Another examination a few months later showed 224 000 platelets and a bleeding time of twenty two and one-half minutes The coagulation time was three minutes (Lee and White)

The patient was last seen in his home in San Pedro, Dec 28, 1928 He weighed 83 pounds (38 Kg), having gained about 10 pounds (4 5 Kg) during the previous six months, and had an appearance of general physical well being which had always been lacking at former examinations At this time the tourniquet test was negative Coagulation of blood was complete in five minutes At room temperature the clot did not show any retraction after twenty-four hours and only one or two drops of serum were evident. Another specimen placed in an incubator over night showed the clot adherent to the test tube, except at one small place near the top but with considerable serum above

In November a tooth had been extracted, followed by moderate oozing of blood for two days A blood smear made shortly afterward revealed the following differential count *polymorphonuclear neutrophils, 46* per cent, eosinophils, 1, basophils, 0 small lymphocytes, 44, large lymphocytes, 4, and monocytes, 5 per cent Additional blood smears made at the same time were forwarded to Dr *Faber at the Stanford University School of Medicine*, who reported that the red and white cells were normal in appearance, and the platelets very abundant Counts of seventeen fields, made by Miss Hadden of the department, showed a ratio of one platelet to 12 1 red blood cells On the basis of a 5,000 000 red count the platelets would number 413,000 The platelets stained well and varied considerably in size The great majority were within normal limits (from 2 to 3 6 microns) but a few abnormally large forms were seen, one of which was 7 microns in diameter

COMMENT

A critical analysis of the cases mentioned makes a proper classification extremely difficult It is at once apparent that they do not correspond to any of the well known types of purpura, and that they have much in common for one who is "seeking similarities and not diversities" The purpuric manifestations associated with a normal platelet count and the heredofamilial factor are fairly constant observations, whereas a considerable variation exists in the bleeding and coagulation times, as well as in the clot retraction phenomenon The confusion is partly due to the lack of uniform laboratory methods for these determinations and also *to the fact that a definite variation occurred in many* of the individual cases at subsequent examinations The condition must carefully be differentiated from other forms of purpura, *particularly from those rare* cases of familial Werlhof's disease reported by Hess[1] Rosenfeld[11] and others, and also from hemophilia and *familial telangiectasia*

The etiologic mechanism in this form of purpura is of course not clearly understood but Glanzmann[*] has attributed it to a *functional insufficiency of the platelets* The inability of the clot to retract is due, he feels, to the absence of a ferment, the so-called retractozyme, or its presence in a less active form *The isolated* platelets obtained from one of his patients caused coagulation of a platelet-free normal plasma, but clot retraction did not occur A similar experiment is mentioned by Buckman,[4] who caused coagulation of blood from a hemophiliac patient with a suspension of platelets from another of his patients The blood coagulated in fifteen minutes, which corresponded exactly to the coagulation time of a normal control, but no mention is made of clot retraction Too much emphasis should not be placed on the ability of the clot to retract, as this phenomenon is not always constant in normal persons Glanzmann has offered additional evidence based on histologic studies, to support his theory of pathologic platelets He has found abnormalities in the size of the platelets and also alterations in the protoplasm and granules Pyknosis and other signs of degeneration were frequently encountered Some of his conclusions are rather severely criticized by Frank[12] in his discussion of pseudohemophiliac conditions He states that he has seen similar pathologic disturbances in other diseases, and feels that some of the changes are the result of faulty staining technic The belief that these cases are closely related to essential thrombopenia and do not form a separate disease entity is considerably strengthened by a report of Krömeke[13] He has described a grandmother, her three daughters and two grandchildren, all with typical purpura hemorrhagica but with great differences in the platelet counts The grandmother had a definite thrombopenia (28,000), one of her daughters and one grandchild had a moderate reduction of platelets (110,200 and 109,300), another daughter and grandchild had normal counts (228,000 and 234,000, respectively) This series forms an important link between essential thrombopenia and so-called thrombasthenia and points to a common etiologic factor which is not a mere numerical reduction in the number of circulating platelets

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[11] Rosenfeld A \ *Idiopathic Purpura with Unusual Features* Arch. Int. Med. 27 465 (April) 1921

Little can be said concerning the treatment employed in the various cases Blood transfusion has proved of great aid in tiding the patient over the severe attacks of bleeding Little and Ayres felt that roentgen irradiation of the liver and spleen was of value, but the evidence is not conclusive The bleeding from the nose greatly diminished in those patients whose tonsillar tissue was cauterized or removed Splenectomy is probably not indicated, although it is far too early to attempt to settle this much mooted question It is encouraging that most of these patients stand operative procedures extremely well and that with advancing age there is a tendency toward natural improvement

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[12] Frank E Die haemorrhagischen Diathese in Schittenhelm s Die Krankenheiten des Blutes und der Blutbildenden Organe Berlin Julius Springer 2 289 1925

[13] Krömeke F Zur Frage der hereditaren haemorrhagischen Diathese (Thrombasthenie) Deutsche med Wchnschr 48:1102 (Aug 18) 1922

Senior Students and Medical Economics—For over twenty years, in season and out I have advocated the need of instruction to all senior students of our medical colleges on the problems of medical economics and this for two reasons—first in order that they may serve society better and, second in order that they may themselves prosper economically Such a course of instruction, in order to serve its purpose must fulfil both of these requirements Dr Donham of the Harvard Graduate School of Business Administration expressed the purpose of that school in the following terms The school is attempting to teach men to work out their own business relations in ways which contribute to social progress" Medical students need such a course of instruction much more than do business executives.—Ochsner, E H Some Medical Economic Fundamentals, *J Indiana M A*, June 15, 1929

VISCEROPTOSIS [*]

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Descriptive anatomies have been the source of the misinformation that a theoretically normal body exists in which a definite type and relationship of organs are found and deviations from which have been described in footnotes as anomalies or abnormalities Until comparatively recent years students have attempted to find in the living the same topographic relationships as were found in the cadaver The bodies used as the basis for descriptive anatomic studies were usually well developed, muscular ones which, in a measure, fitted in with the types previously described Professors of anatomy require clear-cut anatomic material to serve best for their lectures and illustrations

The necessity for recognition of various normal types of the genus *Homo*, each differing from the other in certain major features, is now quite apparent

The use of the x-rays in studying the skeleton, body cavities, viscera, positions and organic relationships has revealed many marked deviations from the previously described anatomically normal Mills[1] defines nature as "an infinite number of variations"

Radiology has opened up a new chapter in the history of man's study of man It is giving us a mass of information, not yet correlated, from which will finally be deduced the specifications of certain broad, general types of bodies which may be looked on as normal This is important Lack of appreciation of the differences that exist between these normal groups of physiques is the basis for numerous grotesque therapeutic measures that habitually fail

In 1917, Mills[1] submitted a classification of body types which may with profit be kept in mind when dealing clinically with aberrations in posture, visceral position and tone with their attendant train of symptoms

As dominant types Mills describes the hypersthenic group, characterized by a large body short thorax, long abdomen, extensive breadth of chest and upper part of the abdomen, with a relatively small pelvis well padded with fat, and the asthenic group characterized by frailty of build, long narrow shallow thorax, with a short, thin abdomen and relatively wide and capacious pelvis

To the hypersthenic and asthenic groups, Mills added the sthenic and hyposthenic subdivisions In each of these there is a fairly constant relationship between the body habitus and the visceral form, position, tone and motility A thorax or abdomen with definite limitations can, of necessity house organs only of certain form

Pathologic conditions may result in gross abnormality in the form and position of viscera, and lesser degrees of pathologic conditions may result in commensurably smaller variations in visceral topography Unless some standard of values is recognized it is impossible to determine the abnormal Before assuming such a condition as visceroptosis, it is obviously necessary to study large numbers of subjects, this is being carried on in several clinics at the present time Meanwhile, in the pursuit of this information numerous suggestive features stand out The stomach of the hypersthenic and sthenic person is found fluoroscopically to lie well up under the ribs, in fact, all of the organs are high, whereas in the hyposthenic and asthenic types the viscera are in lower position and may be found in the upper pelvis

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[*] From the Lankenau Hospital Medical Clinic

[1] Mills, R W Relation of Bodily Habitus to Visceral Form Tonus and Motility Am J Roentgenol 9:155 (April) 1917

The tone and motility of the former are always greater than in the latter groups

The diagnosis of a prolapsed organ cannot be made with any degree of accuracy before the type of body habitus is considered Thus in a hypersthenic person, a hypotonic and dilated stomach with the lowermost point of the greater curvature located in the upper pelvis may be designated as a stomach below par for that individual and likely to contribute its share in the production of dyspepsia Studies of the asthenic type confirm the belief that only with an extreme degree of atony and the greater curvature deep in the pelvis is a diagnosis of elongated stomach permissible With these basic rules of diagnosis in mind, and admitted to be only arbitrary a further and valuable diagnostic procedure, fool-proof in a way, is the therapeutic test

In a given individual, the abdominal organs are maintained in position by

1 Mesenteric and ligamentous attachments
2 Intra-abdominal fat.
3 Intra-abdominal pressure
4 Tonus of the voluntary and involuntary musculatures
5 The shape of the abdominal cavity

1 The fundamental importance of the mesentery is rarely emphasized Through it pass the nerves, blood vessels and lymph vessels to the gastro-intestinal tract The mesentery probably plays only an inferior role in bearing the direct weight of the bowel but, on tension, reflex stimulation of the efferent nerves produces an increase in the tonicity of the external abdominal musculature, which assists in retaining the integrity of the visceral positions Fixed points of the gastro-intestinal tract exist at the diaphragm, second portion of the duodenum, and the ascending and descending colon by ligamentous and mesenteric attachments Incomplete development, usually of the right colonic fusion, may facilitate excessive mobility Abnormal bands or adhesions due, in some instances, to over development may cause stasis with subsequent distention of the large bowel

2 Intra-abdominal fat acts as a cushion for the viscera, reinforces the mesentery, and effectively protects the mesenteric vessels, it accentuates the lumbar anterior curve of the spine and, by generous deposits in the pelvis, diminishes the capacity of the lower portion of the abdomen If the fatty tissue is absorbed and the patient loses weight, a general sagging of all the abdominal viscera takes place

3 The intra-abdominal pressure may be conceived as a negative force existing between a recoiling tube within an osseomuscular chamber It may be influenced by

(*a*) the size of the tube, regulated by its peristalsis, muscular tone and contents,

(*b*) the tonicity of abdominal musculature, and

(*c*) probably also by the intestinal gases as well as by the atmospheric pressure

4 Tonus of the voluntary and involuntary muscles is important to optimal function of the viscera Relaxation of the external muscles leads to a general sagging of the abdominal organs On stimulation of the pacinian corpuscles, nerve end-organs in the mesentery, by increase in tension on the mesentery, a reflex is set up—the muscular mesenteric reflex—which causes the external voluntary muscles to contract and thereby relieve the tension on the mesentery, a condition produced by changing from the prone to the upright posture Loss of tone of the involuntary musculature of the bowel from stasis, debilitating diseases, pregnancy or fatigue accounts for the elongation of the gastric outline and dilated large bowel seen fluoroscopically

5 The shape of the abdominal cavity determines the limits of the various visceral positions In the hypersthenic body type the upper part of the abdomen is capacious and the diaphragm is high, the lumbar vertebral prominence is marked and the pelvis is relatively small The asthenic body type has a cramped upper portion of the abdomen with less prominence of the lumbar vertebra, and the pelvis is large The axis of the upper part of the abdomen, in the hypersthenic type, forms approximately a right angle with the axis of the pelvis In the asthenic type this angle is obtuse It is, therefore, obvious that the limitations of the body cavity must be kept in mind when visceral locations are considered abnormal

Ptosis of one or more abdominal viscera is a common finding in clinical experience Three classes of patients exist (1) those with ptosis and no symptoms, (2) those with ptosis in which symptoms are caused by another condition, and (3) those with ptosis and symptoms due to ptosis Textbooks have described the hereditary and acquired cases, the former being an asthenic habitus, the latter following prolonged disease, pregnancy or inanition

In 100 cases now under observation in the Lankenau Medical Clinic, twenty-seven were classed as congenital and seventy-three as acquired cases of ptosis

The organs involved were stomach 91 per cent, transverse colon 88 per cent, right kidney 3 per cent, left kidney 1 per cent, and liver 2 per cent of the 100 cases The age incidence is given in the accompanying table

Age Incidence in a Series of One Hundred Cases

Years	Number of Cases
From 10 to 20	4
From 20 to 30	32
From 30 to 40	16
From 40 to 50	24
From 50 to 60	20
60	4

Thirty-two males and sixty-eight females were studied The complaints of these patients refer principally to the gastrointestinal tract The majority had been previously diagnosed as "neurasthenic" Consideration of the ptosis did not come up until all of the usual "symptomatic" and "empiric" therapeutic measures had failed to relieve Forty-six patients bore operative scars previous to our study

Examination revealed that 69 per cent were undernourished and 15 per cent were overweight, forty cases showed an erythrocyte count below four million, twenty-two cases exhibited hypotension and in twelve the blood pressure was elevated, sixty-two cases showed hypo-acidity and eighteen cases hyperacidity of the gastric secretion as found by the two-hour fractional meal

Eighty-three patients complained of constipation and in all of these the colon was found, fluoroscopically, to be deep in the pelvis A mobile cecum was noted in eighty-two cases

The gastric wall was hypotonic in thirty-three and hypertonic in five cases Enlargement of the thyroid gland existed in nineteen patients, and eight showed evidence of arthritic changes

It is not our thesis to prove that visceroptosis is the underlying cause of these multitudinous conditions Ptosis is not a disease, but it may result from a debilitating illness In certain cases it may be regarded as an index of the functional ability of the gastro-intestinal tract It is not to be denied that patients are seen who possess a marked prolapse of the stomach, transverse colon or right kidney, but whose complaints are not directly related to those organs It is our belief that the best therapeutic result will be obtained only if the physician in charge will attempt to return these viscera to their best functional position, at the same time that the principal lesion, infection or intoxication is being eradicated Too often the ptosis is disregarded The patient becomes a chronic complainer, or has been one for an extended period of time

Five years ago a middle aged, unmarried, tall, well built, muscular woman walked into the Lankenau Hospital and applied for relief from sick headaches, indigestion, gaseous eructations, constipation and fainting spells from which she had occasionally collapsed Her further statements revealed that she had made the rounds, so to speak, of doctors' offices and tried numberless remedies without relief, the symptoms meanwhile becoming more severe Examination of the patient revealed a moderately relaxed abdominal wall, not at all of an advanced degree Fluoroscopically the stomach showed an atonic wall with the lower border of the greater curvature 3 cm below the level of the anterior superior spine, and the transverse colon lay in the pelvis At the time no attention was directed to the vertical elongation and relaxation of the gastric wall or the malposition of the colon Numerous tonics, sedatives and injections were tried, all without avail The patient continued to have indigestion and weak spells Complete reorganization of the treatment was indicative On surveying the observations made of the case once more it was decided to apply an abdominal support, order abdominal massage with elevation of the foot of the bed, and the patient was given a special diet and requested to lie down for approximately forty-five minutes after each meal Promptly on institution of this regimen the symptoms disappeared and within two months the patient was in better health and spirits than at any time during the preceding three years

I cite this case as an example of a large group of persons suffering from a functional disturbance of the gastro-intestinal tract which is characterized by atony and lengthening of the gastric wall with or without coloptosis The case is stated in such terms because relief of symptoms is unlikely to result if the stomach and colon are not retained in a position compatible with their optimal function

Much has been written pro and con concerning downward displacement of the various abdominal viscera It is generally accepted that a so-called visceroptotic habitus or "maladie constitutionelle" exists In our experience, numerous patients in whom an elongated stomach or dropped colon exists would not ordinarily be placed in such a category

A certain confusion and uncertainty appears to exist with regard to descriptive terms used in reference to the condition at hand Ptosis indicates prolapse or downward displacement of an organ, the liver, kidneys, spleen, transverse colon or stomach may one or all be fallen There is an important point of difference regarding the stomach compared with the other viscera Except for the colon, the other organs are solid The stomach is a muscular tube capable of distention or contraction Gastroptosis, as such, is rarely if ever present, that is to say, this distensible tube is permanently fixed at two points, namely, at the cardia, where it is maintained by ligaments and the diaphragm, and at the fixed second part of the duodenum Gastroptosis is, in truth, a sagging of the antrum and pylorus, which, in extreme cases, may extend downward into the pelvis At the same time there is hypotonicity of the muscular gastric wall, as readily seen fluoroscopically This diminution of tone is, to my mind, important in the production of symptoms

DIAGNOSIS

Ptosis of one or more abdominal viscera is easy to diagnose The outstanding event in the history appears to be the failure of all therapeutic measures previously tried and the number of times the patient has been pronounced "nervous" Not infrequently, operative scars are found Prolapse of viscera may occur in the sthenic or hypersthenic habitus, but more frequently is found in patients with the characteristic hyposthenic or asthenic constitution

TREATMENT

Treatment should be directed toward eradication of all foci of infection, return of the prolapsed organs to their optimal functioning location, and a general toning up of the entire body musculature

Elimination through the bowels, kidneys, skin and lungs is indicated

Rest is essential In the upright posture, tension on the mesentery occurs in these patients This is relieved on lying down, therefore, it is prudent to advise rest following each meal

At no time should the stomach be required to entertain a large meal Accordingly, four, five or even six feedings, each small in amount, are found to facilitate digestion and are always more kindly received Fluids are taken only between meals

Massage of the abdominal muscles with daily exercises to strengthen them is important in retoning the patient

Medicines play a minor role in treatment An irritable pylorus is relaxed by small quantities of belladonna, a sensitive gastric mucosa is allayed by the alkalis, malt and cod liver oil contribute splendidly to upbuilding, and the sedatives of bromide and phenobarbital quiet injured nerves

Abdominal supports are useful and often uncomfortable The majority of belts have been found to be totally incompetent The proper support should be fairly comfortable and at the same time lift the prolapsed viscus to its proper position It must be easily applied In our experience the Page support, consisting of a firm oval leather pad held over the pubic region by a curved rod passing posteriorly over each iliac crest and connected to a back-brace consisting of two short vertical rods, has proved satisfactory It is wise to check up the position of the stomach and transverse colon at frequent intervals, following barium ingestion with the belt in position Minor adjustments are often necessary As soon as the tone of the voluntary and involuntary musculature is sufficiently improved, the stiff belt should make way for an elastic and inconsequential binder so that there may be no pressure atrophy from the Page support

In approximately 5 per cent of patients, adhesions bind the viscera in their malposition so that operation must be considered Likewise, incomplete fusion of the right colon with subsequent gross prolapse may finally warrant surgical intervention It might be wise to emphasize that visceroptosis should always be considered before, and not after, surgical measures

In our series of 100 patients, eighty-two improved under treatment Of the remaining eighteen patients, two were afflicted with pulmonary tuberculosis, four with heart disease, three with nephritis, and one with thyroid disease, five had some form of pelvic disorder

This investigation and survey was considered advisable for the reason that visceroptosis is commonly found and many physicians maintain that it is of no clinical significance I believe that the therapeutic test disproves this assumption I would recommend a reexamination of every patient looked on as a chronic complainer, in whose behalf the diagnosis of some form of ptosis had not hitherto been considered

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THE EFFECTS ON HUMAN BEINGS OF A TWELVE MONTHS' EXCLUSIVE MEAT DIET

BASED ON INTENSIVE CLINICAL AND LABORATORY STUDIES ON TWO ARCTIC EXPLORERS LIVING UNDER AVERAGE CONDITIONS IN A NEW YORK CLIMATE *

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The question of minimal and optimal protein requirements has received considerable research attention in recent years There is now very little disagreement among students of nutrition as to what these requirements are On the effects of a high protein dietary, however, not only do opinions of authorities differ but the results of carefully controlled experiments show considerable variance It is a traditional belief that a high protein intake leads to high blood pressure, arteriosclerosis or nephritis Among physicians it has almost become a dietetic dogma to reduce or eliminate entirely the intake of meat whenever diets are prescribed Unless proper interpretation is given to certain present-day investigations on protein metabolism, including the results of the experiment reviewed in this paper there is danger that the dietetic pendulum will swing too far in the opposite direction

The Eskimos have lived on a high protein dietary as far back as records go Thomas, who recently made a careful study of their health status, found very little evidence to indicate that hypertension or nephritis was more prevalent among Eskimos than among low protein feeders Several years ago it was my privilege to make an exhaustive clinical survey of Vilhjalmur Stefansson, the arctic explorer who had lived for seven years on an exclusive meat diet The result of this study indicated that such a dietary had left no physical or clinically proved stigmas, and that while Stefansson was living on nothing but meat and fish (including fat) his health was at its maximum

The data presented quite naturally gave a jolt to our accepted ideas of protein metabolism Some of the critics maintained that the arctic cold was responsible for this high protein and fat tolerance, others that it was due to vigorous exercise in the open air As a matter of fact, summer heat comparable to our own was experienced for a part of each year in the arctic circle and, since practically a tropical temperature exists in the houses and beneath the fur clothing, this explanation was untenable Exercise likewise was not a factor, since the exclusive meat diet worked just as well during months of sedentary existence

In order to answer some of the queries which Stefansson's experience provoked and to throw additional light light on the effects of an exclusive meat diet, Stefansson and Andersen (who spent three years with him in the arctic) consented to go on an exclusive meat diet for a year and to be intensively studied from every clinical angle

The studies which this paper capitulates have just been completed Monographs [1] detailing the results of these investigations, including basal metabolism, chemical analysis of the blood and urine and fecal bacteriology, are appearing under the names of the respective investigators

DETAILS OF INVESTIGATION

This research extended over a period of twelve consecutive months and was divided in point of time, location and experimental subjects as follows

1 Stefansson—The studies on Stefansson at the Russell Sage Institute of Pathology at Bellevue started, Feb 13, 1928 From that date to February 26 he came to the hospital for all meals but he did not stay at the hospital continuously until February 26 After the completion of preliminary studies he was placed on an exclusive meat diet on February 28, and for two months intensive metabolic and laboratory studies were made on him Then, for a period of ten months he was at large attending to his affairs but reported once each month for clinical and laboratory check ups The concluding meat diet studies lasted one week. Dietary control was supervised in his home and he reported to the hospital for metabolism and blood studies He was under observation for two weeks following the completion of the meat diet. His meat diet experiment ended March 8, 1929

Dietary Notes—After preliminary studies on a mixed diet Stefansson was put on an exclusive lean meat diet for the purpose of studying the effects of an excessively high protein minimal fat dietary Stefansson predicted that he would be ill in a few days, judging by his past experience in the arctic, and such proved to be the case. Although this experiment was planned for but four days, in the evening of the second day he became nauseated and developed some of the discomfort, lethargy and weakness of the knees which he experienced on an enforced lean meat diet in the North. The next day all the symptoms became intensified and diarrhea developed This part of the experiment was of course, stopped and by adding fat in tasteful quantities he fully recovered within two days He lost

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* This work was done under the direction of a committee of scientists under the chairmanship of Dr Raymond Pearl of Johns Hopkins University from contributions made by the American Institute of Meat Packers.

[1] Two short articles on the metabolic results have already been published McClellan W S Spencer, H J Falk E. A and Du Bois E F Clinical Calorimetry XLIII A Comparison of the Thresholds of Ketosis in Diabetes Epilepsy and Obesity, J Biol Chem 80:639 (Dec) 1928 McClellan W S and Toscani Vincent Clinical Calorimetry XLIV Changes in the Rate of Excretion of Acetone Bodies During the Twenty Four Hours, ibid 80:653 (Dec.) 1928 Lieb C. W and Tolstoi Edward Effects of an Exclusive Meat Diet on Chemical Constituents of the Blood Proc. Soc Exper Biol & Med. 26 324 325, 1929 The following articles are in the course of preparation McClellan W S and Rupp V R Note on the Efficiency of Absorption from the Intestinal Tract with Meat Diets Rupp V R Toscani Vincent, and McClellan W S Nitrogen Calcium and Phosphorus Balances of Two Men on an All Meat Diet Rupp V R Analyses of Meat Nitrogen Fat Calcium and Phosphorus The reports of the basal metabolism of the specific dynamic action of foods will be made by Drs McClellan Spencer and Falk Certain phases of the blood chemistry and the response to the injection of dextrose will be published by Dr Edward Tolstoi Dr John C Torrey and Dr C W Lieb will report on the studies of the intestinal flora

2 Kg in the first ten days Following the acute enteritis just described he developed a stubborn constipation coincident with a craving for special foods, particularly calves' brains (10 per cent ether soluble substance) and bacon. He ate heartily of this combination for several meals but this overcompensatory diet resulted in two days of nausea and severe diarrhea By adjustment of the lean fat ratio a normal gastro-intestinal state returned, and no other diet complications developed during the year's experiment

2 ANDERSEN—Preliminary studies were made on Andersen from Jan 6, 1928, when he entered the hospital up to January 25 He then left the hospital for eight months, reporting at monthly intervals for clinical and laboratory surveys Concluding meat diet observations lasted three weeks in the hospital and he was under observation for two months following the conclusion of the meat regimen

Dietary Notes—Andersen did not suffer such digestive upsets as Stefansson, mainly because no all-lean meat (high protein) observations were made on him Andersen lost weight steadily while on the mixed diet from the day he entered the hospital (60 Kg) and continued to lose during the first week he was on the exclusive meat diet (57 8 Kg) He then began to gain weight steadily and within sixty days after the start of the experiment his weight was 60 5 Kg, thus exceeding his initial weight by 0 5 Kg His weight fluctuated slightly during the interhospitalization period, being 58.4 Kg at the beginning of the concluding calorimetric studies Andersen ended his continuous meat diet, Jan 24, 1929 From then to January 31, inclusive, he was placed on a high fat diet with moderate protein From February 1 to February 7 inclusive he was on the same diet plus 35 Gm. of carbohydrate in the form of thrice cooked cabbage From February 8 to February 14 he was on the regular exclusive meat diet. During these periods he was studied intensively from the clinical, laboratory and calorimetric standpoint

February 13, he developed a slight cold He had a dextrose tolerance test on February 15 after which he was supposed to go on his general mixed diet In the evening of this day he developed a chill with rise of temperature The next day he developed signs of a lobar pneumonia of the right lower lobe with a temperature of 104 F and a rusty sputum This proved to be type II pneumonia Dr Felton's concentrated polyvalent pneumonia serum was then intensively given with an immediate drop in temperature, which reached normal within a few days Andersen made an uneventful convalescence Aside from signs of acute intoxication on the fourth day of the disease he showed very little of the toxic symptoms of other patients in the hospital at this time with type II infections While Andersen was in the hospital there were many cases of type II pneumonia in the wards and his was possibly a case of cross infection When one considers that approximately 50 per cent of these patients died it is noteworthy that he survived It may be of interest to know that Andersen during his seven years residence in Florida, has had a low resistance to head colds, having a succession of them each winter He had only one cold during the past year and that was caught during an epidemic of grip in the hospital seventy days after he started on the meat diet. He had a very mild attack and only through the insistence of his physician did he go to bed, where he remained but two days His physician caught the grip at the same time and was confined to bed ten days with a very severe attack Though this clinical incident does not prove anything, it is at least an interesting fact that, in two subjects contracting grip at the same time and apparently from the same source the resistance of the one on the exclusive meat diet should apparently exceed that of the subject on the mixed diet.

Stefansson, while traveling through the Middle West this winter contracted a very mild and short attack of grip in a community where the grip was very severe and the mortality great Of like interest in connection with the subject of resistance and diet is the experience of Stefansson during his explorations in the Far North Having contracted a severe case of typhoid in the arctic in a region remote from medical help and where he was forced by circumstances to eat nothing but canned food, his case was given up as practically hopeless Stefansson, however, while in the third week of his typhoid, demanded that he be taken by sleigh to a hospital 140 miles distant En route he ate nothing but raw fish He reached the hospital in two weeks fully recovered from his typhoid and with a gain of 20 pounds (9 Kg) in weight

These citations are not made for the purpose of proving the resistance-promoting merits of an exclusive meat diet but merely as interesting clinical observations in a field of knowledge demanding exhaustive research and a judicious appraisal of facts and theories

CLINICAL SUMMARY

Weight—Stefansson lost 2 5 Kg during his year on the exclusive meat diet Some of this loss he considered excessive weight Andersen lost 3 Kg up to his attack of pneumonia

Caloric Intake—Stefansson averaged about 2,650 calories a day, 2 100 calories consisting of fat and 550 of protein Andersen averaged about 2,620 calories a day, 2,110 calories consisting of fat and 510 of protein Carbohydrate in the meat varied between 20 and 50 calories a day

Blood Pressure—Stefansson maintained a blood pressure of 105 systolic and 70 diastolic during the entire course of his diet Andersen's blood pressure was 140 systolic and 80 diastolic at the beginning of the diet and 120 systolic and 80 diastolic on the completion of the year's diet

Vitality—In neither subject was there a decrease in physical vigor Both led sedentary lives throughout the experiment No subjective nor objective signs or symptoms of a decrease in physical vigor was observed The same holds true of their mental vigor

Heat Tolerance—Andersen affirms that he endured last summer's heat, if anything, better than during former years

Appearance—Both men appeared ruddier at the end of the experiment than at the beginning

Hair—Andersen noted that his hair stopped falling out shortly after the meat diet was started and that the progressive loss has been checked up to date

Teeth—In neither case has there been any deterioration of the teeth The dentist who has watched these two subjects throughout the experiment noted a greatly increased deposition of tartar on Stefansson's teeth while on the meat diet than formerly This is interesting in view of the low calcium content of meat There was also a notable absence of pyorrhea in both subjects

Bowels—Andersen's bowels have remained normal throughout the diet regimen and required practically no catharsis Stefansson, who has had a tendency to constipation while living in civilized communities, found no increase in this tendency while on the meat diet An interesting point in this connection is that in both these men the stools were nonodorous and there was no evidence of gaseous distention or flatus at any time The stools were somewhat below average size and usually formed

Pulse, Temperature and Sleep—There was no variation from the normal and usual

COMMENT ON DIET

Of interest in this dietary regimen was the apparent absence of monotony the subjects rarely craved for other food than meat products What little calcium they had was obtained from eating the ends of ribs and gristle, averaging about 0.1 Gm. a day. The average water which they drank contained 0.01 Gm. of calcium per liter. Most of the meat eaten was cold storage meat. For a period of two months in the early part of their regimen they had freshly killed meat. During the remainder of the experiment their meat was ordinary, refrigerated, butchers' meat. The meat was usually boiled or stewed, the inside being left rare. Raw bone marrow was eaten as dessert at times early in the experiment but never during the last six months of it. Very little salt was added to the food by Andersen. No eggs were taken by Andersen during the year. Stefansson sometimes used them on journeys when meat was not available. Neither milk nor its products were used. Coffee and black tea and water were the sole beverages and, of course, unsweetened. About 1,500 to 1,800 cc. of fluid was consumed a day. Usually four meals were taken by Stefansson but three by Andersen each day. The food was always relished and complete satiety resulted after eating. No headaches or digestive symptoms, other than those noted as the result of eating lean meat, were experienced. Both these men were ideal experimental subjects and science owes much to them for their whole-hearted cooperation in enduring so patiently the numerous annoyances incident to the complicated and frequent tests made on them. They were psychologically fit for the experiment since, having had years of experience in living on this diet, they had no mental hazards as to its course or outcome.

The diets on which these two experimental subjects have been for the past year have not been particularly high protein diets, being but 30 or 40 per cent above the average protein intake. Their protein intake amounted to between 100 and 140 Gm. a day. The remaining calories were derived from fat which was equal to about three fourths or four fifths of their caloric intake. Andersen showed little evidence of an adaptation to the utilization of the higher percentages of fat. Stefansson appeared to develop an increased utilization to the high percentages of fat. The highest ketosis noted was in Andersen, the subject who weighed least. Stefansson, who is a better nourished type, tolerated the fat better than Andersen.

MISCELLANEOUS DATA

Metabolism.—The basal metabolism of these subjects showed a uniform variation between minus 10 and minus 20 per cent by the Aub-Du Bois standards. There was a slight rise in metabolic rate of 5 per cent during the first month. It is therefore noted that meat diets do not produce any striking elevation of metabolism.

Weights.—Andersen weighed 60 Kg. at the start. At the end of one month he weighed 58.5 Kg., at the end of the second month he weighed 60.5 Kg., at the end of the year's meat diet, 58 Kg., after recovery from pneumonia 57 Kg. and at this writing, three weeks after recovery from pneumonia, he has regained his original weight.

Stefansson's original weight was 72.5 Kg. At the end of the first month it was 68 Kg., at the end of the second month it was 69, and at the end of the year's meat diet 69.4 Kg. From April, 1928, to March, 1929, there was no change in weight. Stefansson's concluding weight after the calorimeter studies had been completed was 69.7 Kg.

Effects on Kidneys.—1. No albumin, casts or blood was found at any time during the course of the experiment.

2. A red test on Andersen showed 55 per cent excretion at the beginning of the experiment, 62 per cent after recovery from pneumonia after completion of a year's meat diet. (The red test was not carried out on Stefansson.)

3. Urea clearance showed 100 per cent plus.

These tests would indicate an ability on the part of the kidneys to hyperfunction while on a meat diet.

4. Roentgen examination of the kidneys before and at the conclusion of the meat diet did not show any change in size.

Mineral Metabolism.—As Aub's work indicated that an acid diet increases calcium excretion and calcium drainage, one would suspect that subjects on a meat diet, which is an acid diet and is likewise low in calcium, would in a year show a calcium deficiency. The increased amount of tartar on Stefansson's teeth and the lack of evidence of lowered blood calcium offers an interesting field for speculation. There was no decreased density in roentgenograms of the hands at the end of the experiment when compared with the hand of a man on a general diet.

Chemical Analysis of the Blood.—The chemical composition of the blood was little affected except for a slight increase in uric acid and a temporary lipemia. The latter occurred only after unusual amounts of fat were taken. Nonprotein nitrogen, urea, uric acid, creatinine, sodium chloride, sugar, carbon dioxide combining power, serum calcium, plasma protein, albumin (plasma), globulins, total protein (plasma), albumin globulin ratio, and cholesterol were studied before the meat diet and after an exclusive meat diet for twelve months, and no noteworthy changes were discovered. Of interest was the fact that the dextrose tolerance test, consisting of the ingestion of 100 Gm. of dextrose, developed in Andersen a typical diabetic curve resulting in the spilling of sugar for the two following days.

Observations on Intestinal Flora (by Dr. John C. Torrey.)—No blood appeared in the fecal specimens and rarely any mucus, the latter occuring once in the case of Andersen and twice in that of Stefansson. The stool reactions were generally definitely acid—between p_H 6.0 and 6.8—with an inoffensive, slightly acid odor.

The whole meat diet in both subjects caused a simplification of the intestinal flora with a marked reduction both in types and in total numbers of bacteria. The acidophilus, enterococcus and streptococcus types were practically eliminated. The *B. coli* group increased greatly numerically at first but later decreased and for a time in one subject (Andersen) were largely replaced by *B. proteus*. Hemolytic *B. coli* increased in both during the first one to three weeks, then disappeared. No increase in virulence of colon strains occurred. *B. welchii* at the start was present in both subjects in higher counts than usual for normals, and during the first period a great increase occurred. Later the count fluctuated but was generally high. There were very few putrefactive spore-bearing anaerobes at any time. The diet did not appear to introduce them or encourage their growth.

This meat diet did not cause the development of a putrefactive flora in Stefansson and in Andersen only so far as the late appearance of *B. proteus* (noted first after about one year on the diet).

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NEPHROSIS A CRITIQUE *

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Twenty-four years ago Friedrich Müller[1] suggested the word "nephrosis" as a term for renal degenerative changes in contrast to "nephritis," which he limited to inflammatory and proliferative lesions of the kidney Since then there has been an active discussion among pathologists as to how far degenerative lesions should be regarded as part of the inflammatory process, defining inflammation as the reaction of tissues to injury of various sorts, chemical, physical and bacterial This discussion continues, and some pathologists use "nephritis" to include various renal lesions in very large measure, if not entirely, degenerative in character, while others, at present the larger group, distinguish between nephritis and nephrosis as did Friedrich Müller, and some even speak of such various forms of nephrosis as simple nephrosis, febrile nephrosis, necrotic or necrotizing nephrosis, sublimate nephrosis, barbital nephrosis, arsphenamine nephrosis, fat nephrosis, lipemic nephrosis, lipoid nephrosis, syphilitic nephrosis, nephrosis of pregnancy, amyloid nephrosis, diabetic nephrosis, cholemic nephrosis and hemoglobin nephrosis The use of the term nephrosis to indicate degenerative renal lesions seems to have played a useful part in pathology as a descriptive term, but just the best way to fit it into the concept of Bright's disease or chronic nephritis (there is no other satisfactory term if "nephrosis" is accepted, unless one says "nephritis and nephrosis") remains undecided, though it is recognized that there are renal lesions essentially degenerative in nature, entirely different from what is seen in glomerular or other types of nephritis or as a result of various known poisons and toxins It is this sort of a lesion that the pathologist finds usually associated with what many clinicians call nephrosis

Beginning about a decade and a half ago there has been an increasing utilization of the word "nephrosis" for a condition presented by a well defined clinical group of patients, and to this term have been added various modifying adjectives, such as true, genuine, lipoid and chronic This form of nephrosis is described as having the following important features insidious onset, marked edema, decreased basal metabolism, oliguria, marked albuminuria, decreased blood proteins with relative increase in globulin reversing the usual albumin globulin ratio, lipoidemia (hypercholesteremia), good phenolsulphonphthalein excretion, no increase in nonprotein nitrogen of blood, cylindruria but no hematuria, doubly refractile lipoid droplets in urine, and normal blood pressure Our present knowledge of this clinical syndrome is largely the result of the work of Munk,[2] Volhard,[3] Fahr,[4] Epstein,[5] Govaerts[6] and others The condition occurs both in children and in adults A number of observers regard this clinical syndrome as a general metabolic disturbance rather than essentially a disease of the kidney analogous to chronic nephritis or Bright's disease and have expressed this idea by the use of such terms as diabeticus albuminuricus (Epstein[5]) lipogelose (Kollert[7]), diabetes lipoid-proteinicus (Lowenthal[8]), physicochemical modification of body colloids (Munk[2])

If one turns to the literature, one finds that the support for this description of nephrosis is in very large part clinical Reports of cases, grouped clinically as nephrosis, followed through to death with postmortem study of the kidneys, are relatively few That there are patients with the clinical syndrome of nephrosis no one can doubt, though they make up a relatively small percentage of patients with renal disease Just how they progress is not so certain Some die after a relatively short period of disease, usually as the result of infection and predominatingly pneumococcus infection Such cases in adults are reported by

Volhard and Fahr,[9] 1914 1 A boy aged 15 years, duration, fifteen months, appendicitis followed by pneumococcus peritonitis 2 A man, aged 22 duration, seventeen months pneumococcus peritonitis and bronchopneumonia 3 A girl, aged 15 years, duration not very clearly stated, probably about two months pulmonary tuberculosis and pneumococcus peritonitis (kidneys showed amyloid with glomerular lesions)

Bock and Mayer,[10] 1920 A woman, aged 43, duration, three months, pneumococcus peritonitis

Vandorfy,[11] 1921 A man, aged 24 duration seven months, probably syphilitic, pneumococcus peritonitis

Stotz,[12] 1922 A man, aged 51 starvation edema during war one and one-half years later, onset of nephrosis, duration, five months, pneumococcus pleurisy

McNee,[13] 1922 1 A woman, duration, fifteen months (glomeruli described as intracapillary glomerulitis) 2 A woman aged 39, duration nine months, acute pelvic inflammation (glomeruli as in his first case and fair degree of interstitial tissue proliferation)

Fahr[14] 1925 1 A youth, aged 19 duration, six weeks (possibly of syphilitic origin), empyema 2 A woman, aged 38 duration, ten months, lung abscess (Fahr described degenerative (1) and hyaline (2) lesions in glomeruli)

Major and Helwig[15] 1925 A man, aged 34 duration, nine months (autopsy limited to removal of kidneys)

Eckstein,[16] 1926 A girl, aged 14 years duration, five months, probably syphilitic peritonitis with Gram + diplococci (kidney showed amyloid and beginning Schrumpfniere')

Murphy and Warfield[17] 1926 A man, aged 21, duration, sixteen months, apparently no terminal infection, but autopsy limited to small abdominal incision

McElroy,[18] 1927 A youth, aged 17, duration, thirteen months tonsillitis

Govaerts and Cordier[19] 1928 A woman aged 22, duration, about twelve months pneumococcus in blood culture, pulmonary thrombosis (Glomeruli larger than normal with nuclear

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* Read before the Association of American Physicians Atlantic City N J May 7 1929

[1] Muller Friedrich Verhandl d deutsch path Gesellsch Meran 1905

[2] Munk F Ztschr f klin Med **78** 1 1913 Med Klin **12** 1019 (Sept 24) 1047 (Oct. 1) 1073 (Oct. 8) 1916 Virchows Arch f path Anat **226** 81 1919 Med Klin **21** 184 1925 Nieren Erkrankungen ed 2 Berlin Urban and Schwarzenberg 1925

[3] Volhard Die doppelseitigen hematogen Nierenerkrankungen Berlin Julius Springer 1918

[4] Fahr Deutsches Arch f klin Med **125** 66 1918 Virchows Arch f path Anat **239** 32 1922 chapter on Pathologic Anatomy of Morbus Brightii page 176 subpart on Nephrosen page 172 in Henke and Lubarsch Handbuch der speziellen pathologischen Anatomie und Histologie Berlin Julius Springer 1925

[5] Epstein A A Am J M Sc. **154**: 638 (Nov) 1917 Parenchymatous Nephritis J A M A **69** 444 (Aug 11) 1917 Am J M Sc. **163**: 167 (Feb) 1922 Thyroid Therapy and Thyroid Tolerance in Chronic Nephrosis J A M A. **87**: 913 (Sept. 18) 1926 Arch. f Verdauungskr **44**: 31 (Aug) 1928

[6] Govaerts P Compt. rend Soc. de biol **89** 678 680 1923 Bull Acad. roy de méd de Belgique 1924 p 161, **8** 33 1928 Govaerts P and Cordier R Ibid. **8** 510 1928

[7] Kollert V Ztschr f klin Med **97** 287 (Sept) 1923 Klin Wchnschr **5** 441 (March 12) 1926 Kollert V and Starlinger W Ztschr f klin Med. **104**: 44 1926

[8] Lowenthal K. Beitr z. path Anat. u. z. allg path. (Ziegler s) **79** 497 (Feb 15) 1928

[9] Volhard and Fahr Die Brightsche Nierenkrankheiten Berlin Julius Springer 1914 p 96

[10] Bock and Mayer Med Klin **16**: 101 1920

[11] Vandorfy J Med Klin **17**: 656 (May 29) 1921

[12] Stotz Med Klin. **18** 1376 1922

[13] McNee J W J Path. & Bact. **25** 425 (Oct) 1922

[14] Fahr in Henke and Lubarsch Handbuch der speziellen pathologischen Anatomie und Histologie Berlin Julius Springer 1925 p 224

[15] Major R H and Helwig F C Bull Johns Hopkins Hosp **36** 260 (April) 1925

[16] Eckstein A Klin. Wchnschr **5** 1787 (Sept 17) 1926

[17] Murphy F D and Warfield L M Lipoid Nephrosis Arch Int Med **38**: 449 (Oct) 1926

[18] McElroy J B Nephroses J A M A. **89** 940 (Sept. 17) 1927

[19] Govaerts and Cordier (footnote 6 third reference)

proliferation, leukocytes in capillary loops, interpreted as glomerulitis)

Löwenthal,[8] 1928 1 A woman, aged 29, duration, three months, blood culture, *B pyocyaneus*, ulcerative colitis 2 A man, aged 21 duration, four months, bronchitis, erysipelas 3 A girl, aged 16 duration, from twelve to thirteen months acute *parotitis (drained) (In 1926 Lowenthal*[20] *also reported* two cases in children, aged 8¼ and 4¼ years)

Summarizing these, there have been reported eighteen adults with clinical course and postmortem renal observations consistent with the diagnosis of nephrosis In these reports the average duration of the disease has been 8 8 months in these patients from onset of edema to death In fifteen of the eighteen cases at the end there was evidence of acute infection, and of these, in eight there was infection with the pneumococcus The average age was 25 7 years, the oldest, 51 Males and females were equally affected In children, purposely omitted on account of my lack of experience with children and their diseases, except for age the reports seem to indicate a similar pathologic process with tendency to infection often with the pneumococcus, as the immediate cause of death after a not long duration of illness One wonders about these patients, just how much of the pathologic picture has been determined by the elements of bacterial infection and what would have been the appearance had the disease not been terminated after so brief *a course brief in comparison with the* majority of kidneys that we have for study from patients with chronic nephritis

The cases just cited have furnished the opportunity for pathologic study of nephrosis, that term being used in its more narrow clinical sense Besides these cases, surprisingly few in number when one considers the great interest in the subject, the larger majority of patients with nephrosis must have either recovered completely or progressed under some other clinical picture to an ultimate death, since so very few autopsies have been reported in the literature Clinical reports indicate frequent recovery, but the literature is devoid of much in the way of follow-up of these cases in later years to see whether *their apparent cure* was complete or not Some of these patients, after a varying time, die and show extensive amyloidosis This happened in one of our patients, who clinically more than any other of our patients showed the features of nephrosis There are reported also cases which do not heal but progress with a clinical picture shifting from that of nephrosis to subacute or chronic glomerular nephritis, and at death these show a more or less granular definitely atrophied kidney (nephrotische Schrumpfniere) Mason[21] reports such a case and Volhard and Fahr[9] include two of them This has happened in cases at our clinic

Some authors describe in their cases of nephrosis definite glomerular lesions and believe that, even in these patients dying early with renal lesions essentially degenerative in nature, there are evidences of glomerular lesions similar to those found in the earlier stages of glomerular nephritis (Govaerts and Cordier McNee, possibly McElroy) Then there are described mixed cases in which clinically and pathologically there are evidences of both nephrosis and nephritis, cases which seem to progress along the lines of losing the clinical evidences of nephrosis and gaining more and more of the clinical features of glomerular nephritis In my experience this is what usually happens Many of the patients that progress with signs pointing to glomerular lesions give a history of onset indicative of an acute nephritis subsequent to some sort of an acute infection Three patients with an early stage of hemorrhagic nephritis in our clinic have developed the nephrosis syndrome

As one reviews the various reports of patients with the clinical syndrome of nephrosis, one gains the impression that two features are strikingly common to all of these *patients in the* earlier stages, edema and very marked albuminuria, and that the rest of the features of nephrosis come later When the picture is fully developed, *an almost constant* feature *is* lowered blood protein, which is a reversal of the albumin globulin ratio of normal individuals When one recalls the edema of *starvation and that occasionally seen in* pernicious anemia in the stage of severe anemia, there might seem to be a correlation between the lowered protein of the blood and the edema, an idea that numerous observers have expressed in various ways and for which there is some evidence already adduced

With this possibility in mind, Barker and Kirk working in my clinic under the supervision of O'Hare, have kept the blood protein of dogs low for a long time by very frequent bleedings, preventing anemia by returning the washed red blood cells to the just bled dogs This work *will be reported* later by Barker and Kirk Suffice it to say here that these dogs developed edema, low basal metabolism, low blood protein with reversal of the albumin globulin ratio, and increased blood lipoids

In my own clinic we have had, under the leadership of O'Hare, an especial interest in chronic nephritis (Bright's disease) for seventeen years During that period we have had opportunity to observe patients with edema and marked albuminuria and to follow the course of their disease So far we have had but one autopsy that gave the renal changes fairly consistent with *nephrosis, as described by the various reporters, in the* sense of tubular degeneration without glomerular lesions, but this patient failed to show the typical clinical picture of nephrosis All of our patients who had extensive edema of the nephrotic type and who died have shown glomerular lesions, either those usual to glomerular nephritis or an amyloidosis of the kidney including the glomeruli One case already reported[22] may be cited briefly as an example

A woman aged 60, was *admitted to the Peter Bent Brigham* Hospital Aug 31 1917, where she remained until her death, Oct 9, 1918 She had diphtheria three months before admission Shortly after this her eyes were puffy and her legs began to swell Shortly her abdomen swelled Never after this was the edema lost Her blood pressure was 165 systolic and 95 diastolic She had ascites and this was pseudochylous in type *when tapped, in all on fifteen occasions* The urine always contained a large amount of albumin, hyaline and granular casts and red blood cells from rare to very many *The basal* metabolism ranged from minus 41 to minus 35 (eight observations) In the last few weeks of life the phenolsulphonphthalein excretion fell and the blood urea nitrogen rose, otherwise renal function was good except for salt and water Autopsy showed a marked degree of chronic glomerular nephritis

Similar to our experience appears too to have been the experience at Mount Sinai Hospital, where Epstein had worked and Baehr[23] last spring made the statement I would like to agree with the previous speaker and call attention to the fact that the clinical diagnosis of nephrosis is being made altogether too frequently Every case of glomerulonephritis has a certain nephrotic element At the Mount Sinai Hospital in New York, where we have studied nephrosis for a long time, we have only once encountered a pure nephrosis in an adult at autopsy Every other case clinically diagnosed nephrosis has at autopsy presented characteristic evidences of glomerulonephritis The one time nephrosis occurred in an adult was in a syphilitic In children we do see pure nephrosis not infrequently In my experience they are usually superimposed on a chronic pneumococcus infection in the upper respiratory tract Eventually most of these children die of a pneumococcus infection of their ascites

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[20] Lowenthal K. Virchows Arch f path Ana 261 109 1926
[21] Mason E H Internat. Clin. 1 163 (March) 1926

[22] Christian Oxford Medicine New York, Oxford University Press American Branch 3 658 1921 3 692 of revision of Dec 1 1924
[23] Baehr Am J Path. 4 632 1928

A study of the recent literature, along with my own clinical experience and the observations on dogs of Barker and Kirk during the current year, have led me to adopt the following conception of nephrosis as a clinical condition

1 Given a considerable and prolonged protein deficit in the blood, edema, lowered blood protein, relative increase in blood globulin at the expense of albumin, hypercholesteremia, and lowered basal metabolism will appear, not always in the same order and possibly in varying degree

2 The most common cause for such blood protein deficit is marked and prolonged albuminuria

3 The cause of such albuminuria is a renal lesion

4 Often this renal lesion is obviously in the glomeruli, but in many reported cases, mainly those patients dying of infection relatively early in the course of the disease, the glomeruli show very slight or no demonstrable lesions, while there are extensive degenerative changes in the tubules For the latter, knowing what we do of renal physiology, and believing that the actual daily amount of albumin in the urine in many of these patients is in excess of what could possibly come from degenerating renal epithelium, I am of the opinion that the source of the albuminuria is in the passage of albumin through the walls of blood vessels, most probably, glomerular capillaries, a functional change in a colloid membrane of which the microscope gives no evidence Obviously this explanation is mere surmise

5 Holding these views, there seems to me to be no justification for regarding nephrosis as other than a variety of kidney disease, a form of chronic nephritis (Bright's disease)

6 Clinically it seems to me that nephrosis may be used as the name of a syndrome such as defined by Munk, Volhard, Fahr, Epstein and others, but when it occurs it does not mean that a definite and single form of pathologic lesion will be found in the kidney, but rather that there may be a degenerative lesion of the tubules with normal appearing glomeruli there may be amyloidosis, there may be glomerular lesions that will progress into typical chronic glomerular nephritis Furthermore, all of these lesions may be found in patients who never showed the syndrome of nephrosis

7 While I can offer no objections to such a clinical use of the term nephrosis, I can see no great advantage of it over the term I have used in a very simple clinical classification of nephritis, namely subacute or chronic nephritis with edema If used clinically I think it should be used as the name of a syndrome occurring during the course of chronic nephritis (Bright's disease) If it is helpful to the pathologist to use nephrosis for degenerative lesions as suggested by Müller, obviously there can be no objection, only as clinicians I think we should recognize that we may be unable during life to predict accurately whether the pathologist is going to find more or less nephrosis or less or more nephritis, in other words, we are still in the same predicament in which we have always been, unable to use a pathologic classification of nephritis that can be applied easily and accurately in the clinic

8 The important practical contributions of Munk, Volhard, Fahr and Epstein and the others has been in emphasizing the protein loss in these cases and the need of liberalizing the diet for renal cases, so that it contains a liberal protein content to replace the loss of albumin from the blood, so long as renal function will permit of its utilization without retention of nonprotein nitrogen

9 In this group there are certain, though few, patients with a definite cause for the renal disturbance, syphilis

10 One gets the impression that what has been added to the clinical picture in recent years has resulted from the application of newer clinical methods giving more exact data in regard to processes formerly known or at least suspected, and that after much writing about it not a great deal more of importance is known today than was understood by clinicians of the past under the terms parenchymatous nephritis, amyloid disease of the kidney, syphilis of the kidney, the lipemia of certain cases of nephritis, and the pasty pallor, edema and languor of some forms of nephritis Extensive tubular degeneration in certain types of large white kidney has been known for a long time Older descriptions of amyloid kidney and syphilitic nephritis, allowing for differences in terminology and lack of some new laboratory methods, correspond extremely closely with those of "lipoid" nephrosis Diminution of concentration of plasma protein was noted by Bright[24] in 1836, as was the milkiness of the serum, 'if the blood is drawn it is often bufted or the serum is milky and opaque, and nice analysis will frequently detect a great deficiency of albumin" Changes in the albumin globulin ratio have been the subject of study for many years[25] Opalescent serous effusions were pointed out a long time ago Lowered basal metabolism had to await clinical methods for its determination, and this seems to be about the only new feature that recent investigations have added to our knowledge of the group of patients with extensive edema of renal origin This lowered basal metabolism does not seem to be other than part of the picture Evidence for its relation to deficient thyroid function is meager and in my opinion, it should not be considered analogous to myxedema due to a lesion reducing thyroid function

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[24] Bright Guy's Hosp Rep 1 338 1836

[25] Mya and Viglezio (1888) cited by Csatary who also reports decreased albumin globulin ratio Deutsches Arch f klin Med 48 358 1891

Contributors to Obstetrics and Gynecology—If a young physician were suddenly thrust backward forty years one of his strangest experiences would be to miss so many names with which we are familiar in our branch of medicine There was no Stroganoff to systematize the use of morphia in eclampsia no Watkins to show us how to cure a cystocele, no Wertheim to teach us how to do a panhysterectomy for cancer no Potter to improve the technic of version and to enlarge its indications no Rubin's test for patency of the tubes no Titus or Thalheimer to demonstrate the advantages of the intravenous treatment of the toxemia of pregnancy with dextrose and insulin no Kielland Barton or Piper with their new models of forceps —Hirst B S The Progress of Teaching and Practice in Gynecology During the Last Four Decades *Am J Obst & Gynec* February 1929

THE STABILITY OF DIGITALIS AND ITS PREPARATIONS*

HARVEY B HAAG, M D

AND

ROBERT A. HATCHER, M D

NEW YORK

Many have sought to determine the cause of deterioration in preparations of digitalis, and the discovery of one or more factors that are concerned in the deterioration has caused certain manufacturers to claim complete stability for their preparations The accumulation of a large number of specimens of digitalis leaf and preparations of many types, some of which have been standardized during the past twenty years, afforded us an opportunity for making a comparison of the rate of deterioration in a wide variety of these preparations

A few references to the literature will suffice to indicate prevalent opinions and we shall then present only those results of our experiments which are of practical interest in therapeutics, leaving the details of the experiments to be published elsewhere All our tests of activity, except a few with rapidly eliminated fractions, were made by the method described by Hatcher and Brody[1]

Focke[2] reported that a specimen of leaves that contained 12 per cent of moisture lost about three fourths of its activity within less than three months, he stated that equal deterioration occurred within a year in other specimens having from 6 to 8 per cent of moisture, and great deterioration when there was only 3 per cent of moisture present Since nearly all digitalis leaves contain moisture unless kept with excessive care Focke's results would appear to indicate that practically all digitalis undergoes rapid decomposition

Hale[3] investigated several factors concerned with the variability of digitalis and its preparations, including the influence of time He found that leaves 8 years old containing 9 1 per cent of moisture were of the same activity as recent English leaves While the activity of these specimens had not been determined previously, Hale's results do not lend any support to Focke's contention

Gold and De Graff[4] examined four specimens of digitalis that had been examined previously, one five years, one four years and two more than two and one-half years before None showed deterioration, though they had been kept in corked bottles without special precautions

Houghton and Hamilton[5] examined thirty-eight specimens of previously standardized extracts, fluidextracts and tinctures, and reported that they had deteriorated at rates varying from an average of 4 per cent a year during six years to that of 10 per cent a year during three and one-half years They were inclined to the view that a high alcoholic content favors stability

Pittenger and Mulford[6] examined fifteen tinctures and reported deterioration in every specimen except one varying up to 75 per cent in seven months, and Hamilton,[7] in attempting to refute the conclusions of Pittenger and Mulford, examined six specimens of tincture He found much lower averages of deterioration, but one of his specimens was 43 per cent less active after six months

Hale examined eight preparations in 1908 and in 1910 and found deterioration varying from 4 3 per cent in the strongly alcoholic to 33 3 per cent in the weakly alcoholic He reported that digitol, containing 30 per cent of alcohol, and digalen, which contained 7 5 per cent, had deteriorated more rapidly than the official tinctures, which contained approximately 70 per cent He also examined three tinctures, one 8 years old, one 3 years and one of 2 weeks and found them to be of equal activity Haskell, Daniel and Terry[8] in 1922 examined four tinctures that had been tested in 1917 Their table shows that the average activity of these was exactly the same in 1922 that it was five years before

Some have sought to avoid oxidation by replacing the air in the container with an inert gas Weak hydrochloric acid decomposes certain constituents of digitalis, such as gitalin, rapidly even at body temperature, and Hatcher and Eggleston[9] found an old acetic extract of digitalis to be practically inert Heat used in making the infusion causes changes in some of the active principles of digitalis, with some loss of activity Some years ago a pharmaceutic manufacturing firm was asked to discontinue the marketing of ampules of ouabain on the ground that they became almost inert within a few days, and a prominent pharmacologist wrote to one of us that he had been *informed that infusion of digitalis* became almost inert after a single day

Hatcher and Eggleston obtained a specimen of digitalis leaf from E R Squibb & Sons and examined it in 1913, at which time it was at least 25 years old The leaves were powdered at that time and the powder has been kept in a corked wide-mouth bottle, without special precaution, during the past sixteen years We find that this powder, now at least 41 years old, has the same activity that it had in 1913, and that it is more active than many fresh specimens obtained recently

A specimen of powdered digitalis from the crop of 1905 was sent to this laboratory in 1906, and during the past twenty-three years it has stood on a shelf in the pasteboard carton in which it was received Its activity at present is about 70 per cent of that of the average digitalis and there is no reason to suppose that it has undergone any deterioration, but, unfortunately, it was not examined at the time it was received

One of us prepared an infusion of digitalis, Feb 5, 1918 heated it to the boiling point, and with it completely filled small bottles into which corks were forced, and the bottles were sealed with paraffin The activity was determined shortly after the infusion was made, after an interval of a year, after an interval of about two years and five months, and after an interval of nearly eleven years No deterioration could be detected within the first two years and five months, at which time a fraction of this infusion was used clinically by Dr Cary Eggleston[10] The apex rate of a patient after rest in bed was 145 and the radial rate was 72 He received a total of 150 cc of the infusion within twenty-four hours, causing a prompt fall of the apex rate to 68 with a total disappearance of pulse deficit, showing that the infusion exerted the full therapeutic effects After an interval of nearly eleven years the activity had fallen to about one half of the original, but larger doses still produce the typical digitalis action

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* From the Department of Pharmacology Cornell University Medical College

[1] Hatcher R. A and Brody J G The Biological Standardization of Drugs Am. J Pharm. 82 360 (Aug) 1910

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[5] Houghton E. M and Hamilton H C. The Pharmacological Assay of the Heart Tonics Am. J Pharm 81 461 (Oct) 1909

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[7] Hamilton H C. The Deterioration of Digitalis Extracts J Am Pharm. A. 7 153 (May) 1918

[8] Haskell C. C Daniel D S and Terry G S Deterioration of *the Tincture of Digitalis* J Am. Pharm A 11 918 (Nov) 1922

[9] Hatcher R. A and Eggleston Cary Observations on the Keeping Properties of Digitalis and Some of Its Preparations Am. J Pharm 85 203 (May) 1913

Another specimen of infusion was prepared and bottled in the same way, Feb 24, 1921 The bottles were wrapped in manila paper and have been kept in the laboratory without special care since that time When tested, Nov 15, 1928, the infusion had lost about 65 per cent of its activity during seven years and nine months It was stored during the winter in an overheated room almost directly over the furnace, and when tested in April, 1929, it was about one-fourth as active as when prepared

It was used clinically, through the courtesy of Dr John Wyckoff, by Dr Harry Gold, to whom we are indebted for the following reports

CASE 1 —H C, a man, aged 62 after several days rest in bed had a ventricular rate of 100, and a pulse rate of 78 He received 570 cc of the infusion during four days The ventricular rate and pulse rate dropped to 54 with marked general improvement and the excretion of 4,300 cc of urine during the last two days The patient s weight decreased by 10 pounds (4 5 Kg) On the next day 150 cc of the infusion was given, with complete digitalization, including nausea

CASE 2 —R. A., a man, aged 54, after several days' rest in bed had a ventricular rate of 126 and a pulse rate of 90 He received a total of 680 cc of infusion during six days The ventricular rate and pulse rate dropped to 84, without other marked improvement

It is especially interesting that this infusion which had deteriorated to one fourth of its original activity exerted the typical digitalis action The activity of 570 cc of the infusion, shown by the biologic test, was equal to that of 14 cc of an average tincture of digitalis, or about the amount usually required to digitalize a patient

A specimen of digitol aqueous (now called "digitos") was received from the manufacturers, April 29, 1922 When examined after an interval of seven years, it was about one-half as active as a specimen of digitol obtained directly from the H K Mulford Company, April 19, 1929 It is about as active as our 11 year old infusion and more active than that made in 1921

A commercial specimen of infusion of digitalis in ampules, which had been kept in this laboratory during ten years or more, was less active than our older infusion, and a little more than that made in 1921 The activity of the infusion in the ampules was not determined at the time they were received, but the results do not lend any support to the view that the infusion kept in ampules is more stable than that kept in a carefully corked glass bottle While no one would think of recommending that infusion of digitalis be kept for many years, the results of our experiments are instructive in that they show that a properly made infusion does not undergo rapid decomposition, and further, that the loss of activity does not involve the development of dangerous toxic substances

Reference has been made to the suggestion that ampules of ouabain (so-called crystalline strophanthin) are unreliable This suggestion was made in 1918, at which time the manufacturers in question were asked to submit specimens of ouabain solution in ampules of soft glass and in ampules of hard glass Some deterioration had occurred in the soft glass ampules at the time that they were received in this laboratory, after eleven months they had lost 90 per cent of their activity The solution in hard glass deteriorated much more slowly and during one year no change was perceptible, but when examined in 1923 after an interval of five years some had lost about 60 per cent of their activity Closely similar results were observed with sterile solution of ouabain which one of us had placed in hard and soft glass ampules and in glass-stoppered flint bottles, Feb 9, 1918 No deterioration occurred in the soft glass ampules in six weeks, in the bottles in three months, or in the hard glass ampules in five months At the present time, all the old solutions kept in soft glass are inert or nearly so some of those in hard glass are about as active as they were six years ago, others are a little weaker Ampules of amorphous strophanthin which were imported more than ten years ago were also nearly inert The results of these experiments show that properly prepared ampules of ouabain in hard glass may be used within a reasonable period with confidence, but that soft glass should not be used

An alcoholic solution of ouabain which had been kept in a glass-stoppered bottle on a shelf in the laboratory during twenty-one years showed a loss in activity of only 20 per cent Ampules of cymarin received, April 13, 1914, have lost about 70 per cent of their activity Ampules of digitalysatum, received in this laboratory in September, 1915 labeled "Stensol-Digitalysatum," containing no alcohol, were practically inert in 1929 This is of especial interest because of the claim that has been made that digitalysatum is especially stable in contrast to the other digitalis preparations, and that no change occurred after a lapse of five years Curiously, a specimen of digitalysatum containing 12 per cent of alcohol which had been kept in an ordinary corked bottle, not sealed, without care since September, 1915, lost only about 30 per cent of its activity in nearly fourteen years, assuming that this was the same as that of a specimen which had been examined shortly after its receipt in this laboratory

Ten ampules of digifolin were submitted to this laboratory by a hospital in February, 1923 The label indicates that the liquid is of the same activity as the tincture of digitalis, but these ampules were apparently less than half as active as the tincture of digitalis then used in the hospital Ampules of digifolin received in June, 1928, were tested in May, 1929, at which time the activity was one-third that of the average official tincture of digitalis Ampules of digifolin received in November, 1916, were tested in April, 1929, and found practically inert It is not remarkable that those ampules which had been kept thirteen years had undergone extensive deterioration, but the results with the other two specimens were distinctly surprising because the advertising received in 1928 contains the statement ' The fact that Digifolin-Ciba does not deteriorate with age is sufficient evidence that deterioration is due to the presence of saponins and other decomposition products These substances have been removed from Digifolin-Ciba "

We do not mean to imply that digifolin-Ciba or digitalysatum is more prone to decomposition than are nonofficial preparations but, on the other hand, the manufacturers' claims of unusual stability are not supported by the results of our experiments

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[10] Eggleston Cary Unpublished data

The stability of digitalis in powder is being utilized in some forty affiliated cardiac clinics of the New York Tuberculosis and Health Association following the institution of the use of standardized tablets in the Adult Cardiac Clinic of Bellevue Hospital six years ago. An amount of digitalis sufficient to supply these clinics at least one year (and probably two years) has been standardized by Dr. Harry Gold and it has been made into tablets of several different sizes, insuring comparable dosage in all the clinics. It is obviously desirable to extend the principle to include all cardiac clinics in the United States in order that studies of dosage in one clinic may be compared with those in others. There is no obvious difficulty in accumulating supplies of the leaf to last more than one year, though it might take more than a year to bring the plan into operation.

Infusion of digitalis in tightly corked and sealed bottles deteriorates very slowly. We do not recommend that it be kept for years, but the clinician can use with confidence the infusion prepared by a properly qualified pharmacist. The preparation does not require any special skill.

There is some evidence that the active principles in the infusion which resist decomposition are precisely those which are absorbed most readily from the gastro-intestinal tract, and that those which decompose are the principles which are poorly absorbed and which contribute little to the therapeutic action of oral doses of the infusion or tincture.

The official tincture of digitalis, containing about 70 per cent alcohol, deteriorates so slowly that the clinician can use it with confidence that it will induce the desired effects in suitable cases, especially since modern practice directs that the dose be increased cautiously, if necessary, until minor toxic symptoms, such as nausea, are induced. With this in mind one may use a tincture of any age in an emergency, with confidence that sufficient doses will induce the typical digitalis action.

There are several causes that contribute to the slow deterioration of the infusion and tincture of digitalis, but the presence of a little air in the bottle is not important. The decomposition is probably due mainly to a constituent of digitalis, since the purified principles in 60 per cent alcohol, and ouabain in 90 per cent alcohol, deteriorated little during many years.

SUMMARY

Six specimens of powdered digitalis have been examined in this laboratory after intervals varying from one to sixteen years, and in no case has deterioration been detected, and it may be added that no one in this laboratory has ever observed anything indicative of deterioration in one of the many specimens of powdered digitalis used.

Powdered digitalis, in tablets or in capsules, is admirably suited for securing uniformity of dosage where individual patients, clinics or groups of clinics are provided with sufficient to last one year or more.

A sterile infusion of digitalis undergoes little change within several months, and deterioration then results solely in diminished activity, not in increased toxicity.

The official tincture of digitalis retains its activity with comparatively little change during several years, and any change that does occur merely calls for a corresponding increase in dosage. The secret of deterioration of liquid preparations of digitalis has not been explained fully, and there is no evidence that any of these preparations are as stable as powdered digitalis kept with ordinary care in a corked glass bottle.

Aqueous solutions of strophanthin, ouabain or other digitalis principles, kept in ampules of soft glass, deteriorate rapidly. Ouabain solution in ampules of hard glass decomposes slowly.

Our investigation lends no support to the contention that any of the digitalis specialties mentioned are more stable than the official digitalis tincture. All liquid preparations of digitalis should bear the date of manufacture.

414 East Twenty-Sixth Street

Clinical Notes, Suggestions and New Instruments

NARCOLEPSY FOLLOWING EPIDEMIC ENCEPHALITIS

GORDON R. KAMMAN M D ST PAUL
Instructor in Nervous and Mental Diseases University of Minnesota Medical School

Narcolepsy as a sequel of epidemic encephalitis is a rare although definite entity described by Economo,[1] Stiefler,[2] Symonds,[3] Adie,[4] and Kluge[5] in Europe and by Spiller[6] in America. As far as I am able to determine, Spiller's is the first and the present one is the second account in American literature of narcolepsy occurring as a postencephalitic phenomenon. Several other cases of so-called narcolepsy following encephalitis have been reported in the literature, but careful examination of the reports shows that these are not cases of true narcolepsy but merely inversions of the sleep rhythm with sleep occurring during the day. Also, many of these cases do not have the characteristic "Lachschlag" which the present one shows.

Narcolepsy was first described by Gelineau in 1880, and since then descriptions of the disease have been extremely inconsistent. It has been called by various names—Singer's "Hypnolepsie," Friedmann's "Kleine gehaufte Anfalle"—and its symptomatology has been confused with everything from hysteria to epilepsy. The most widely accepted concept of the syndrome today is that offered by Gelineau and Redlich,[7] who state that it has two outstanding symptoms. These are (1) periodic attacks of involuntary sleep, and (2) attacks of temporary loss of tone in the voluntary muscles brought on by some emotional stimulus. The attacks of involuntary sleep may come at any time during the day, they last for from several minutes to half an hour, the sleep is quiet and normal, and the patient can be awakened by adequate stimulation. The amount of sleep the patient receives at night does not influence the number of diurnal sleeping attacks, which may occur under the most varied conditions, such as walking, talking driving, plowing playing cards, military or naval duty, and even during coitus.[8] The second symptoms, "loss of tone" attacks, appears when the patient laughs is frightened, or becomes angry. There is a sudden loss of tone in all the skeletal muscles, and the patient collapses without experiencing any disturbance in consciousness. This condition lasts for only a short time, and in a few minutes the patient becomes perfectly normal. Because this phenomenon so often accompanies the act of laughing, German writers refer to it as a "laugh stroke" (Lachschlag).

Literature contains accounts of both 'essential' and "symptomatic" narcolepsy. 'Essential narcolepsy' is the term applied

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[2] Stiefler G Narkolepsie nach Encephalitis Lethargica Wien Klin Wchnschr **40** 1044 1046 (Oct) 1924

[3] Symonds C P Narcolepsy as a Symptom of Encephalitis Lethargica Lancet **2**: 1214 1215 (Dec.) 1926

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[8] Adie W Lecture at Queen's Square Hospital London October 192

Examination—The physical examination revealed a general orange-yellow pigmentation of the skin, most marked in the palms of the hands and the soles of the feet, but no tingeing of the sclerae. The transverse colon was slightly tender The liver came below the costal margin about 3 cm. on deep inspiration, the edge was smooth and not tender The remainder of the physical examination was negative

Laboratory Observations—The blood pressure was 130 systolic and 80 diastolic, the hemoglobin was 88 the red blood cells numbered 4,600,000, and the leukocytes 9,200 The urine was normal, there was no bile, the stools were soft and formed, and bile pigments were present A cholecystogram made by Dr Rose showed normal filling and normal emptying, there was no evidence of calculi A barium enema revealed a normal colon except for a slight spasticity in the region of the transverse portion. The Wassermann reaction was negative, the icterus index was 33.3, and the van den Bergh reaction was negative. A fragility test of the red corpuscles showed initial hemolysis at 0 42, complete hemolysis at 0 34

"Jaundice" that was so unevenly distributed, did not color the sclerae, did not produce bile in the urine, and gave an icterus index of 33.3 and a negative van den Bergh reaction, it was reasoned, could not be a true jaundice Dr Matthews one of our interns, suggested as a diagnosis carotinemia This was in accord with all the observations and was supported by a further history from the patient of an unusual fondness for carrots, at least one cereal bowl full having been eaten every day for three months Removal from the diet of the carotin-rich vegetables and fruits resulted in a marked improvement in one week, and complete disappearance of the pigmentation in six weeks

COMMENT

Carotinemia in an adult is an uncommon condition and of interest only because of the possibility of confusing it with a true jaundice, and the necessity of recognizing its etiology in order that it may be cleared up Pseudo-icterus and xanthosis diabetica are undoubtedly synonymous with carotinemia It is not a disease but a harmless pigmentation which rapidly disappears with the elimination from the diet of the carotin-rich foods

104 North Oak Park Avenue.

RUPTURE OF THE RECTUM DURING PROCTOSCOPIC EXAMINATION *

Charles Goldman M D Brooklyn

My interest in rupture of the rectum during proctoscopic examination was aroused when the case presented here came under my care

History—A white man, aged 44, a city fireman, married was seen, Feb 5, 1929, with the complaint of severe constipation, loss of appetite, general weakness, and a history of an attack of influenza three weeks previous to his visit. A general examination disclosed myocardial degeneration, a systolic pressure of 104 and a small quantity of albumin in the urine.

A proctoscopic examination showed a normal empty rectum A diagnosis of postinfluenzal exhaustion and constipation was made and the patient was given an anticonstipation diet, told to take liquid petrolatum, and advised to rest and to spend a few hours each day in the sunshine.

Because the constipation was not entirely relieved by the liquid petrolatum and the diet, or for some reason not known to me a nurse who was in attendance on another patient in the same building began daily colonic irrigations On the advice of the nurse, a neighborhood physician with whom she was acquainted was called for medical advice.

February 20, the patient was ready to return to work fully recovered from his illness The patient, at the request of the physician, went to his office for a final examination When the physician, after a cursory general examination introduced a proctoscope, the patient shrieked in pain and collapsed. He was rushed to his home and put to bed with a diagnosis of gas pains After twenty-four hours of agony and several colonic irrigations under the physician s orders, the patient was transferred to a local hospital

The essential facts of the further history are taken from the hospital history chart

The patient died, February 27, as a result of general peritonitis

The admission diagnosis was acute peritonitis, February 21, the chief complaint was pain in the abdomen. There was no history of blood in the stools or vomiting of blood The urine was normal

Examination—The patient lay in bed with the legs flexed, he appeared acutely ill, and had an anxious expression on his face. The head was of normal contour The pupils were equal and reacted to light and in accommodation The throat was clear Examination of the chest revealed shallow respiration but was otherwise negative The abdomen was flat, and there was rigidity over the entire abdomen which was very tense at the lower part. Palpation could not be done owing to the rigidity and tenderness

Operative Record—A diagnosis of perforated viscus was made. During anesthesia the pulse was poor The patient vomited black material.

1 The intestines throughout were severely inflamed and distended and covered with a fibrinous exudate.

2 The gallbladder, serous coat, was covered with petechial markings

3 The abdominal cavity was filled with a large quantity of seropurulent substance.

4 The left pelvic space was filled with a large quantity of greenish white gelatinous pus

5 There was a perforation about the size of a silver quarter (25 mm) on the right anterior surface of the rectum about 6 inches (15 cm.) above the anus

Operative Procedure—Repair of the perforation was done, with drainage of the abdomen and of several loops of the intestine

Autopsy—The essential observations were myocardial degeneration, congenital cystic kidneys, bronchopneumonia, and normal bowel, except for a perforation of the anterior wall of the rectum about 6 inches from the anus

COMMENT

On the advice of Dr Edward C Martin of Detroit, I attempted to complete my report by the addition of as many unpublished similar cases as I could collect I soon found that the nature of the case prevented any great assistance from my colleagues One patient, I knew, had come to a Brooklyn hospital within the last year The surgeon in charge was unwilling that I see the chart. However, he supplied me with the following meager description of the case

A man, aged 52, complained of cramps and diarrhea Following proctoscopy, he complained of sudden severe pain, went into shock and rapidly developed diffuse distention of the abdomen with tenderness The patient was brought to the hospital within three or four hours and operated on immediately At operation a diagnosis was made of rupture of the rectum and a colostomy was performed. The patient died No autopsy was obtained

I wrote to a number of men doing proctology and asked them whether they knew of any unpublished cases of rupture of the rectum during proctologic examination Two men replied that they each knew of a case but that they were not privileged to give information for publication. One man replied giving the details of a rupture of the rectum during proctoscopic examination in a case of ulcerative colitis

CONCLUSIONS

1 The normal bowel may be ruptured with comparative ease.

2 The number of cases of rupture of the rectum during proctoscopic examination reported is only a small part of those which occur

3 There is a necessity for insisting on at least as much instruction in the use of a proctoscope as is believed necessary in the use of a cystoscope.

1501 Union Street.

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* Read before the American Proctologic Society Detroit.

ASCARIS LUMBRICOIDES FOUND IN THE CAVITY OF THE HUMAN HEART*

Carl Boettiger, M.D. and Jacob Werne, M.D. New York

The case we are reporting is perhaps of interest more because of its oddity than of any scientific significance the observations may carry.

A woman, aged 65, born in this country, was admitted to the medical service of Dr. McLave at St. John's Hospital, Feb. 18, 1929. The only history obtainable, since the patient was irrational, was from her son. She was a tertipara who had borne her last child forty years before. She had had a swelling of her right leg for twenty years and had recently been complaining of pain in the left lower portion of the chest. She had been treated at the Metropolitan Hospital for ulcers of the leg during the six months prior to her admission to this hospital.

The patient was said to have become acutely ill the day before admission. She developed chills, fever, and the pain in the chest previously mentioned. There was no hemoptysis. Physical examination in the hospital revealed, as significant observations, crepitant rales over both lungs, distant heart sounds of regular rhythm, and elephantiasis of the right leg with ulcerations over the ankle. The temperature on admission was 100.4 F., the pulse rate was 105, and respirations were 38. The chemical analysis of the blood was not remarkable. There were 4,100,000 red blood cells and 11,000 white blood cells, of which 67 per cent were polymorphonuclears and 33 per cent lymphocytes. No eosinophils were found. The urine contained a trace of albumin. Death occurred three days after admission. The clinical diagnoses were pulmonary thrombosis, chronic bronchopneumonia and elephantiasis.

The autopsy revealed cardiac hypertrophy, general arteriosclerosis, pulmonary thrombosis, chronic interstitial pneumonia, passive congestion of the lungs, liver and spleen, chronic interstitial pancreatitis, elephantiasis of the right leg with superficial ulcerations, and ascariasis. The immediate cause of death was pulmonary thrombosis.

The interesting feature of this case was the finding of two yellowish white worms with annular markings, measuring each about 10 cm. by 5 mm. in the cavity of the right ventricle. The first was found in the chicken fat clot in the cavity of the right ventricle and the other was found to extend partly into the pulmonary artery. In view of the clinical diagnosis of pulmonary thrombosis, incision had been made into the right ventricle and pulmonary artery before the removal of the viscera *en masse*. No adult forms of the parasite were found in the intestine, which is the normal habitat of the mature worm.

The heart was markedly hypertrophied but did not show any valvular or endocardial lesions, aside from moderate arteriosclerotic thickening. The myocardium grossly did not show extensive scarring. Both lower lobes and the right middle lobe of the lung were the site of a marked induration and interstitial fibrosis which will explain the finding of old thromboses some with canalization. A fresh clot, adherent and granular, distended the wall of the vessel leading to the right middle lobe.

The worms were identified as forms of *Ascaris lumbricoides*. Because of the unusual finding, a brief survey of the literature on this subject was made, and disclosed several references to the finding of parasites in the cavity of the human heart of which, however, so vague a description is given as to render them unauthoritative.

In this connection, it might be well to call to mind the accepted cycle of development of *Ascaris lumbricoides* in man. The larvae, liberated by the hatching of the eggs in the small intestine, are known to penetrate into the portal circulation. After a brief sojourn in the liver, they pass through the right heart into the lung and undergo further development in the capillaries. From there they pass to the air vesicles, up the tracheobronchial tree, down the esophagus into the stomach and small intestine where they develop into adult worms. These parasites have been found in the biliary passages, in the pancreatic ducts, in the eustachian tube, in the lacrimal duct, in the genito-urinary tract and free in the peritoneal cavity, among numerous other locations. Whether in this case development occurred somewhere between the portal circulation and the pulmonary artery, or whether the adult worm had made its way into the cavity of the right heart, either ante mortem or post mortem, is a speculative question. Suffice it to say that the tendency of this parasite is known, and has been variously reported, to migrate into the most unusual situations in the body.

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* Read before the Queens County Medical Society, April 7, 1929.
From the Pathological Laboratory of St. John's Long Island City Hospital.

SYSTEMIC BLASTOMYCOSIS

Theodore Toepel, M.D., Atlanta, Ga.

The case reported here is of interest on account of the rapidity with which blastomycosis progressed by the direct route along bones from the index finger to the upper arm, and by metastasis to the right lung, then to the left lung and finally to the left kidney.

The history dates back to Dec. 24, 1927, when the patient was pricked by a thorn on the left index finger while hunting rabbits. Papules appeared on all the fingers shortly afterward.

Jan. 12, 1928, a red spot was noticed on the index finger which developed into an abscess. The finger was lanced, January 18, 22 and 25, without relief.

The patient, a married man, aged 37, weighing 200 pounds (91 Kg.), came to me, Jan. 31, 1928. Physical examination was negative except for the infected finger, which was swollen to twice its size and of purplish glistening color. The blood and urine were normal. Roentgen examination revealed osteomyelitis, involving all the phalangeal bones, with considerable bone destruction of the distal phalanx. The finger was amputated, February 1, the second metacarpal bone was removed, March 3, and the arm was amputated at the middle and lower third of the humerus, March 13. The wounds were left open and tincture of iodine was freely used.

February 3 the presence of blastomycetes was definitely established in the pus of the finger and was again found in the hand and in an abscess on the upper arm. The blastomycetes were discovered in the sputum, April 2. This condition cleared up in ten days. When the patient left the hospital, April 25, there was no evidence of blastomycetes in the sputum or in the urine.

Laboratory observations are of interest, as they showed the rapid decline of the red blood cells from 4,960,000, January 31, to 3,800,000 by April 19; the white blood cells rose from 8,400, January 31, to 34,500 by March 31 and then dropped to 23,800 by April 19; lymphocytes dropped from 32 per cent, January 31, to 4, March 26, and then rose to 17 by April 19; polymorphonuclear neutrophils rose from 65 per cent, January 31, to 95, March 19, and then dropped to 81 by April 19; hemoglobin dropped from 92 per cent, January 31, to 75 by April 19. Two blood Wassermann reactions were negative; blood tularemia was negative. Special urine tests for iodine were positive, varying from + to + + +; albumin varied from a trace to + and the microscopic examination showed leukocytes, occasional hyaline casts, pus cells and blastomycetes.

A point of interest in this case is the absence of further involvement of the bone after the last operation, March 13, the metastasis to the right lung, April 2, and a clear lung without blastomycetes, April 15. When the patient left the hospital, April 25, he was able to walk to the automobile. A roentgenogram of his chest, taken at his home town and sent to me about the last of May, showed a large area involved in the left lung and a week later the report sent to me told of the patient passing pus and suffering extreme pain over the left kidney. The patient died, June 22, 1928. The complete destruction of the left kidney was discovered at the autopsy; fibrous patches in both lungs and in the liver were present.

Potassium iodide was given by mouth, beginning February 3, a dosage of 20 drops of the saturated solution, three times a day, being increased daily until the patient was taking 120 drops three times a day. Fifteen grains (1 Gm.) of sodium iodide in 20 cc. of solution was given daily intravenously. Tincture of iodine was freely used in the wounds, which were left open for this purpose, from February 1 to March 13. Laxatives and enemas were given as needed, and sedatives for pain. The stomach and body developed a tolerance for the potassium and other iodides, so that only on rare occasions was the dose reduced or discontinued.

Special Article

ABOLISHING DIABETIC COMA

E P JOSLIN, M D
BOSTON

Diabetes is a chronic disease, but diabetic coma is an acute disease If this fact was recognized, deaths from diabetic coma should cease It is high time they should cease for seven years have gone by since the discovery of insulin and the medical profession has been taught that insulin cures coma unless the patient is moribund Why not abolish diabetic coma forthwith? This is the easiest and surest method of lowering diabetic mortality

The diagnosis of diabetic coma depends largely on a history of its gradual onset The patient has felt sick and looks and acts seriously ill rather than in collapse, as in insulin shock with tremor and sweating, unconsciousness or convulsions Inquiry shows that insulin has been omitted or that the customary dose became inadequate, because the patient broke his diet or contracted an infection Usually nausea, vomiting and pain, simulating serious abdominal disease, have led to the omission of food and in consequence the body has had nothing to live on except its own protein and fat and hence has developed acidosis Gradually increasing restlessness, a deepening of the respiration into air hunger, but without cyanosis, and a change from drowsiness to coma develop, quite in contrast to the symptoms of insulin shock, whose onset is swift, complete and without difficulty in breathing In coma the urine, obtained by catheter if necessary, contains sugar and acid, in insulin shock the urine is sugar free unless it collected in the bladder before insulin began to act

TREATMENT

1 The treatment of coma is the treatment of an emergency and takes precedence over all else The diagnosis once made, insulin should be injected every half hour subcutaneously, from 10 to 40 units or more, until returning consciousness, normal respiration and decreasing glycosuria demonstrate beginning recovery If insulin is given intravenously, it should always be given subcutaneously as well

2 Since the tissues of the patient are dehydrated, a liter (quart) of physiologic solution of sodium chloride should be inserted under the skin at once It is dangerous in coma to trust to the retention of liquids by mouth or the absorption of salt solution by rectum Intravenous injections are satisfactory, if given slowly to avoid distention of the heart

3 The circulation should be stimulated with caffeine sodiobenzoate, 7½ grains (0 5 Gm), given if indicated, every hour for four doses

4 The stomach should be gently washed out so that liquids will be retained Useful liquids are water, broths, coffee, tea, gruels, and often most desirable of all for the first twenty-four hours, two or three glasses of ginger ale or the juice of two or three oranges—in other words, about 50 Gm of carbohydrate Thereafter one may return to the simpler articles of the regular diet, all the while regulating the dosage of insulin by tests of the urine at first every two hours, later lengthening the intervals to four or six hours The dosage is 15 units of insulin for a red test, with Benedict's solution, 10 units for a yellow test, 5 units for a green test, but no insulin if the urine is sugar free With measures such as these, recovery is almost certain unless the complicating disease is of itself fatal

PREVENTION

Prevention is better than treatment The patient should be trained to avoid coma He should be told that he must not omit insulin as long as there is sugar in his urine, no matter whether he eats or does not eat If he has an infection or fever, he must take more insulin and take it oftener He must be made to realize the danger of breaking his diet Ask him to repeat what he is to do if he feels sick

1 Telephone the doctor
2 Go to bed
3 Drink a cupful of hot liquid every hour
4 Get a nurse or a substitute
5 Take an enema
6 Keep warm
7 Have boiled water on the stove ready for the doctor when he comes

81 Bay State Road

Council on Pharmacy and Chemistry

NEW AND NONOFFICIAL REMEDIES

THE FOLLOWING ADDITIONAL ARTICLES HAVE BEEN ACCEPTED AS CONFORMING TO THE RULES OF THE COUNCIL ON PHARMACY AND CHEMISTRY OF THE AMERICAN MEDICAL ASSOCIATION FOR ADMISSION TO NEW AND NONOFFICIAL REMEDIES A COPY OF THE RULES ON WHICH THE COUNCIL BASES ITS ACTION WILL BE SENT ON APPLICATION

W A PUCKNER, Secretary

ISAROL-CIBA—Sulphonated Bitumen, N F—A preparation obtained by dry distillation of bituminous shale The distillate is sulphonated with sulphuric acid and subsequently neutralized with ammonium carbonate. The product complies with the standards for sulphonated bitumen N F

Actions and Uses See Sulphoichthyolate Preparations and Substitutes, New and Nonofficial Remedies 1929, p 398

Manufactured by the Society of Chemical Industry in Basle Switzerland (Ciba Company Inc New York) No U S patent U S trademark 97 007

Isarol Ciba is a reddish brown to brownish black syrupy liquid with a strong characteristic empyreumatic odor It is soluble in water and in glycerin and is miscible with fixed oils and fats It is partly soluble in alcohol or ether and entirely soluble in a mixture of equal volumes of these solvents An aqueous solution (1 in 10) may be faintly acid or faintly alkaline to litmus paper The addition of hydrochloric acid to this solution precipitates a dark resinous mass which is soluble in ether

Incinerate a weighed portion of isarol Ciba the ash does not exceed 0 5 per cent Dry a weighed portion on a water bath to constant weight the loss is not more than 50 per cent

Accurately weigh about 5 Gm of isarol Ciba dissolve in 100 cc. of water transfer to a distillation flask add an excess of sodium hydroxide solution and distil slowly collect the distillate (about 50 cc) in 15 cc of normal sulphuric acid when the distillation is completed, titrate the excess of sulphuric acid with tenth normal sodium hydroxide using methyl orange as indicator the amount of ammonia found is not less than 2 5 per cent Accurately weigh about 1 Gm of isarol Ciba transfer to a 100 cc beaker and add 25 cc of alcohol stir thoroughly filter and wash the filter with a mixture of equal parts of ether and alcohol until the washings are clear and colorless dry the residue on the filter at 100 C cool and wash the filter with 200 cc of warm water slightly acidulated with hydrochloric acid determine the sulphate in the solution by precipitation with barium chloride solution and after washing drying igniting and weighing calculate the results to ammonium sulphate the amount found is not more than 8 per cent Dry about 1 Gm of isarol Ciba on a watch glass to constant weight at 105 C pulverize the dried material and transfer about 0 5 Gm accurately weighed to a nickel crucible add about 9 Gm of sulphur free sodium peroxide, and mix thoroughly place the crucible carefully in a beaker containing cold distilled water which should reach about half way to the top ignite the dry mixture in the crucible by thrusting a red hot iron wire through a hole in the cover of the crucible after complete combustion has taken place tip the crucible and allow the fused mass to dissolve in the distilled water add hydrochloric acid in slight excess heat to boiling and determine the sulphate in the solution by precipitation with barium chloride solution and after washing drying igniting and weighing calculate the results to sulphur the total sulphur should not be less than 10 per cent.

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SATURDAY, JULY 6, 1929

SOME EFFECTS OF FAULTY FAT METABOLISM IN DIABETES

That diabetes mellitus is not a disease of carbohydrate metabolism alone has been demonstrated by the careful quantitative chemical investigations of the past decade and especially by the study of the disease since the introduction of insulin Beginning perhaps as a disturbance of carbohydrate metabolism alone, resulting from hypofunctioning of the pancreatic hormone, it remains a relatively innocuous disease as long as carbohydrate metabolism alone is at fault In this state the patient may live out his normal span of life with a minimal amount of worry about his diet The harmlessness of diabetes in this stage is attested by the frequency with which it exists and remains unsuspected and undetected until its presence is discovered by a chance urine examination It is, indeed, difficult to say how deleterious the mere presence of an increased amount of dextrose in the blood and tissues may be Even this useful and necessary substance may be harmful when constantly present in the tissues in unusually large amount, but there is no very good evidence that a high dextrose content in itself acts deleteriously Blitzsten and Schram,[1] working at the Nelson Morris Institute in Chicago showed that slight changes in the electrocardiogram were present in a fairly large proportion of patients with diabetes and that similar changes could be brought about in dogs by maintaining the blood sugar at a high level during the course of experiments That hyperglycemia alone, the dextrose in high concentration acting possibly as a protoplasmic poison, is a factor in the causation of other changes in the cardiovascular system is problematic That it is a factor at all appears doubtful because of the regularity with which the highly specialized renal tubular epithelium, which is so susceptible to poisons escapes harm until a late period of the disease The high dextrose content of the blood and tissues is probably chiefly responsible for the increased susceptibility of the diabetic patient to infection

Unfortunately, faulty carbohydrate metabolism is all too frequently not the only metabolic abnormality in diabetes As the disease progresses the other two elements, protein and fat, of the metabolic triad are drawn into the field of abnormality and add to and finally even dominate the picture The body, unable to obtain its necessary store of energy from the most readily combustible source, carbohydrate, attacks the protein not only of the diet but also of the body tissues, increasing through the dextrose formed from protein whatever harmful effect the abnormal dextrose content of the tissues may have, and leading, through destruction of body tissues, to the extreme state of inanition sometimes seen in rapidly progressing and uncontrolled diabetes

Of greater evil consequence for the body as a whole than incomplete carbohydrate combustion or excessive protein breakdown is abnormality in the metabolism of fat The latter apparently requires for its complete and normal combustion the fire furnished by the oxidation of carbohydrate, and not merely that supplied by the utilization of dextrose but the hotter blast of glycogenneogenesis and glycogenolysis From the incomplete oxidation of fat there results a series of effects which dominate the clinical picture of diabetes and bring about pathologic and physiologic changes more serious than those which exist before fat metabolism becomes faulty The increase in blood lipoids that leads to macroscopic lipemia was probably known to the observant clinician long before the days of quantitative clinical chemistry The degree and persistence of the lipemia may be a measure of the severity of the disease Rabinowitch,[2] working at the Montreal General Hospital and using the quantitative determination of cholesterol in the blood as a measure of the degree of lipemia, found a direct relation to exist between the progress and prognosis of the disease and the degree of cholesteremia This was true in both children and adults With no or only slight increase in blood cholesterol, the diabetes is usually easily controlled by simple attention to diet, with a moderate increase, insulin becomes necessary, at higher cholesterol levels, both insulin and extreme restriction of diet become necessary and may fail unless the patient is under constant observation

Incomplete combustion of fat leads not only to changes that require chemical methods for their detection but also to morphologic tissue alterations Fatty change of the myocardium is not a negligible factor in diabetes Lipoid storage by the reticulo-endothelial system may lead to histologic changes simulating Gaucher's disease, what the physiologic effect of such storage may be is not definitely known More serious for the patient is the effect of the persistent lipemia on the arterial system Gangrene due to arterial obliteration is one of the frequent complications of diabetes Borchardt[3] has shown that the arterial involvement is not characteristic of diabetes but, in its medial degeneration and calcification and in its intimal atheromatosis with lipoid infiltration, is identical with senile arteriosclerosis of the peripheral arteries The arterial disease occurs at an earlier age and with greater frequency at later ages in the diabetic than in the nondiabetic person, as was pointed out by Borchardt from postmortem studies, and more recently by Morrison and Bogan[4] from roentgenologic study of calcification of the arteries From the similarity of the arterial lesion in diabetes to the experimental aortic arteriosclerosis which can be brought about in rabbits by prolonged cholesterol feeding, resulting in cholesteremia, it is fair to assume that persistent lipemia is an important and determining factor in the arteriosclerosis that leads so frequently to gangrene in diabetes The increased incidence of cholelithiasis noted in diabetes by Joslin[5] is probably to be correlated with lipoidemia Incomplete fat combustion causes its most serious harmful effect in acidosis, which is the dread and often the terminal complication of diabetes That disturbed protein metabolism may be a factor in the causation of diabetic acidosis does not minimize the importance of faulty fat metabolism, since it is probably through the formation of fat from intermediary derivatives that protein may have a ketogenic effect

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[1] Blitzsten E P W and Schram D L Diabetes Electrocardiographic Studies Arch Int Med 36 770 (Dec.) 1925

[2] Rabinowitch I M The Cholesterol Content of Blood Plasma in Diabetes Mellitus A Statistical Study Based on Two Thousand Observations in Three Hundred and Eighty Five Cases Arch Int Med 43:363 (March) 1929 The Cholesterol Content of the Blood Plasma in Juvenile Diabetes A Statistical Study ibid 43 372 (March) 1929

OLD AGE

The natural end of man is old age and death, all of life is but a preparation for this sequel Few there are, indeed, who are willing to accept this dictum of nature The struggle for youth is never ending and the useless hope for the miracle of rejuvenation is perhaps one of the impelling motives that animate the dying man to the end of his existence The subject is one that has stimulated the philosophers since the earliest times The latest contribution comes from Dr Aldred Scott Warthin,[6] professor of pathology in the University of Michigan, who brings to the consideration of the subject a background of knowledge of pathology such as is possessed by few if any others who have written philosophically on old age

The active life of the pathologist involves the study of effects of disease and wear on the structures of the human body He knows all about the body, from its beginning to its end, he knows the variations of its structures in all the periods of life and the anatomic changes that they represent, and he uses this knowledge to interpret with the philosopher's mind the cycle of life Warthin's study of the phenomena of old age and death is really a study of the cycle of human life It is instructive and satisfying not only in its interpretation of the final period of life but also in its description and interpretation of life's whole cycle

Warthin divides life into three major periods the period of evolution, from conception to about the twenty-fifth year in the female and the twenty-eighth year in the male, the period of maturity, to about fifty-five and sixty-five, respectively, then the period of involution, which he would put in the individual who arrives at the end of intrinsic senile death at approximately ninety His thesis is that this is the natural cycle of human life, an intrinsic quality of its vitality, and that it is beyond man's power to modify The biologic purpose of life is reproduction, the continuation of the species for some future destiny, perhaps state of perfection, which we cannot glimpse, and that in this purpose "the individual machine is but a temporary, albeit necessary, incident in the life of the species, and the latter in its turn is also only an incident in the grand march of collective life through the eons "

Throughout the period of growth and even in maturity there are numerous structures, from the tail of the spermatozoon to such major structures as the thymus gland and the ovaries, that are destroyed and disappear when their uses have been fulfilled and their functions are no longer necessary The placenta, Warthin emphasizes, is especially remarkable in its life history "At the time of birth it is a senile structure repeating in detail the essential pathologic tissue changes that later are to appear in the major involution of the mature organism sclerosis of arteries with obliteration, fibrosis of stroma, atrophy and degeneration of the chorionic epithelium, thrombosis, infarction of villi, calcification " He finally clinches his argument with this observation "The point we wish to emphasize is that these early vascular changes are identical in kind with those of the arteriosclerosis of later life, but that they are associated with disuse and not with overuse They cannot be explained on the basis of a *wear-and-tear* or *toxic* etiology " Old age, as revealed by Warthin, is the same purposeful process as the involution of these temporary structures, which he calls the minor involutions "The main differences between the minor and major involutions are those of degree, purpose and the organ or tissue involved The major involution, senescence, affects all of the vital organs and functions, not for any purpose of further growth and evolution, but for the purpose of getting rid of the organism, itself, as a whole This can mean but one thing the individual human machine has fulfilled its function, and, now useless stands in the way of the progressive evolution of the species In other words, the minor involutions take place for the good of the single individual, the major involution for the good of the species"

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[3] Borchardt H Über die Veranderungen der Arterienmedia bei Spontangangran und ihre Beziehung zum Diabetes Virchows Arch f path Anat **250**: 521 1926

[4] Morrison L. B and Bogan I K Calcification of the Vessels in Diabetes J A M A. **92** 1424 (April 27) 1929

[5] Joslin E. P Treatment of Diabetes Mellitus Philadelphia Lea and Febiger 1928 p 540

[6] Warthin A S Old Age New York Paul B Hoeber 1929

This thesis is illustrated by the course of every living thing from the insect which has a cycle of life measured in hours or the flower that lives through one blooming and seeding season, to the cycle of human life, and the cycles of animals which live hundreds of years Obvious as the phenomena of the intrinsic life cycles are, men still try to avoid the idea that the aging process is normal and consider it rather as a progressive disease Many great minds have been possessed, if not obsessed, by this idea Brown-Sequard conceived of age as a disease characterized by the sclerotic changes in the vessels, Metchnikoff carried the idea one step further by explaining these vascular changes by intestinal putrefaction, Victor Horsley by degeneration of the thyroid gland, Lorand and numerous followers of his by degeneration of all the ductless glands To these ideas Warthin comments "The modern scientifically trained biologist, however, cannot look upon senescence and old age as disease processes but regards them as a physiologic entity, the result of involution processes inherent in the organism Age, the major involution, is due primarily to the gradually weakening energy charge set in action at the moment of fertilization, and is dependent upon the potential fulfilment of function by the organism The immortality of the germ plasm rests upon the renewal of this energy charge from generation to generation"

Incidentally, Warthin pays attention to the currently exploited attempts at rejuvenation

> More than thirty years after Brown-Sequard s first experiments made in 1889, in the direction of establishing a relationship between senility and the production of sex hormones by the sex glands this subject has again come to the fore in the active controversy that has centered about the so called rejuvenation produced by testicular transplantation (Voronoff) and ligature of the vas deferens of the testis (Steinach) An immense mass of literature has accumulated, with results so contradictory that positive statements one way or the other cannot be made at this time as to the real status of the questions of the regeneration of sexual activities by these methods of transplantation and ligation If anything does result from these procedures it is quite evident that it is nothing more than a re-erotization, associated with certain evidences of increased energy of the general metabolism There is no evidence that the transplanted testis produces any secretion by living cells what effects are produced are the results of resorption processes The most recent workers agree that the Voronoff operation aside from the psychical influence on the patient is without any specific effect, either on the sexual life or on the process of senescence That there could possibly be any arrest of senility or a rejuvenescence in the broad sense of restoration to youth by the Voronoff operation is in the very nature of things an absurdity Age is a quality characteristic of all cells and tissues in the human body, the cell changes of age are irreversible There has been no evidence offered to show that either the Steinach or the Voronoff operation has abrogated the cell changes of senility or has done anything more than to accelerate in some cases a dying sexual function and certain features of the general metabolism connected therewith The only functions affected are those which depend upon sex hormones In other words the so-called rejuvenation produced by the sex hormones of the transplanted testis or by ligation of the vas is no rejuvenation in any sense of the word but a re erotization wholly
>
> It is a sad commentary upon the mentality and character of the senile human male that he should seek such a re-erotization of his failing function Such a process is not without its hazards, it is another example of the dangers of putting new wine into old bottles as has been shown by the sudden deaths of a number of re-erotized old men The question of the effect of ligating the vas deferens whether due to increased hormone production by the interstitial cells as Steinach supposes, or to the resorption of the products of decomposition of the seminal epithelium, as claimed by other observers, cannot be said to be fully settled, although evidence is accumulating to the effect that the interstitial cells are not glands of internal secretion but represent specialized reticulo-endothelial cells concerned in lipoid metabolism, and bearing in their size and prominence an inverse relationship to the function of spermatogenesis We may therefore be assured that there is no rejuvenescence possible for the senile individual and that the idea of physical rejuvenation is but a myth of ancient lineage disguised in quasiscientific garments

The view of life, old age and death that has been presented may seem depressing to one who longs for physical immortality Some of Warthin's sentences, for example, taken alone, must give this impression "From now on to the end of the third (the last) act he [the actor in the play of life] must portray the picture of gradually flagging desire and failing powers, of disillusionment, fading pleasures, in other words, the portrayal of senescence, until his role ends in ultimate death" But as a matter of fact the philosophy of his book is not pessimistic and its effect is not depressing It has the cheerfulness and optimism that come from a courageous and honest facing of facts "But if age is inevitable, what shall be done about it? Meet it, of course, with courage and common sense, as something in the day's work, not pleasant wholly, but as the final turn of the screw, to be endured with that fortitude that in enduring acquires a quality of satisfaction Moreover, the possibilities of normal old age are great, it is the period of ripeness of experiences and observation, of the contemplation and philosophical evaluation of the world about us, of quiet brooding and the creative possibilities engendered by such Since the mental powers are preserved longer than any other function in senescence, happy is that man who comes into his old age with the capacity for intellectual pleasures fully developed, not in one line alone, but in many, in literature, art, music and science" The book ends with the pertinent and beautiful quotations from Cicero, Harvey and Goethe

> Whatever is natural must be accounted good. When death comes to youth Nature is up in arms and revolts Yet to old men, what is more natural than dying?'—Cicero De Senectute
>
> The eternity of things is connected with the reciprocal interchange of generation and decay, and as the sun now in the east and then in the west completes the measure of time by his ceaseless revolutions, so are the fleeting things of mortal existence made eternal through incessant change and kinds and species are perpetuated though individuals die. —William Harvey De Generatione
>
> Keine Kunst ist s alt zu werden, Es ist Kunst, es zu ertragen.
> —Goethe

To grow old gracefully—that must be enough for any man

THE MECHANISM OF FAT ABSORPTION

Most writers who deal with the mode of absorption of fat from the alimentary tract are at present inclined to the view that it is disintegrated by lipolytic enzymes into fatty acids and glycerol before it penetrates the intestinal barrier[1] As the lymph stream that carries the products of absorption away from the intestine contains neutral fats, it is assumed that a resynthesis of the liberated fatty acids to fat occurs somewhere along the route between the lumen of the bowel and the lymphatic channels One would expect the locus of such changes to be in the cells of the intestinal mucosa Sinclair[2] has pointed out that the resynthesis of fat is assumed tacitly to be simply a reversal of the hydrolysis which takes place in the lumen of the intestine, that is, it is enzymic in nature Nevertheless, attempts to demonstrate in vitro the synthesis of fat from its split products in the presence of hashed mucosa have been either inconclusive or definitely unsuccessful As the mucosal cells always contain phospholipids, it has also been thought that they might be an intermediary stage in the resynthesis of fat from its absorbed constituents If this is true, the phospholipids of the mucosa, and more particularly their constituent fatty acids, should assume the characteristics of the fat that is being absorbed

A test of the hypothesis has been attempted at the University of Rochester School of Medicine and Dentistry by Sinclair The general plan of the experimentation was to feed a fat of pronounced characteristics and to observe the effects on the amount and nature of the phospholipid of the intestinal mucosa The experiments have shown that whereas there is no significant change in the phospholipid fatty acid make-up of the muscular structures either in the intestine or elsewhere in the body during the absorption of fat, there is a pronounced change in the composition but no change in the amount of the phospholipid fatty acids of the intestinal mucosa and of the liver Apparently there is an incorporation of absorbed fatty acids into the phospholipids of the intestinal mucosa According to Sinclair's view, within the epithelial cells of the intestinal mucosa there is a "specific" phospholipid which occupies an intermediary position between fatty acids and neutral fat It is an invariable characteristic that the amount of phospholipid remains constant As soon as fatty acids are absorbed into the epithelial cells, molecules of the "specific" phospholipid react with the free fatty acids (or soaps) to form neutral fat, immediately, however, the phosphoric acid-base complex unites with absorbed fatty acids and glycerol to form phospholipid, thereby maintaining the amount constant The synthesized molecules of neutral fat, being insoluble in the cell fluids, coalesce and form the droplets that are so typical of the process of fat absorption In this manner the neutral fat is made inactive, and the reaction proceeds until all of the absorbed fatty acids have been synthesized into neutral fat

Despite the suggestiveness of the theory of successive fat cleavage and resynthesis prior to and during absorption, one cannot entirely disregard the indications furnished by Mellanby that neutral fat emulsified with bile is freely absorbed from the duodenum into the lacteals of animals in which both the pancreatic duct and the biliary duct have been ligatured Perhaps, after all, some emulsified fat can pass freely into the columnar cells of the villus without previous hydrolysis into fatty acid and glycerol

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[1] The subject is reviewed by Bloor W R Physiol Rev 2 92 (Jan) 1922

[2] Sinclair R G The Role of the Phospholipids of the Intestinal Mucosa in Fat Absorption J Biol Chem 82 117 (April) 1929

MANUFACTURED WEATHER

The so-called present high standard of living, which contrasts so markedly with that of a generation ago, depends for its maintenance on circumstances arising in large scale production and standardization Perhaps no more striking characteristic of modern industry can be mentioned than its gradually developing independence of nature It is only by the assurance of dependable uniformity in supply of raw material, in transportation and in mechanical power that quantity production with the net gain to the consumer has been made possible One of the factors of large importance to many industrial processes is grouped under the term "atmospheric conditions" In a recent account Lindsay[1] has described the rise of the new ventilation engineering and has pointed out the wide application already made of this specialty in a variety of industries

The temperature, moisture and movement of air are of considerable importance in many manufacturing processes, in the past, the location of mills frequently depended on the prevailing atmospheric conditions of a region The situation is now largely changed and, by the use of the newer methods of air conditioning, materials can be dried or made moist, rapidly or slowly at any temperature desired Such procedures as the drying of pottery, the curing of tobacco and the moistening of leather for the uppers of shoes are now in many plants dependable and under perfect control, requiring far less time and floor space and resulting in a more uniform product with less breakage and spoilage than with the older methods The manufacture of rayon, of which 98,000,000 pounds was made in the United States last year, requires controlled air conditions throughout the process The milling of the finest grades of flour involves an accurate adjustment of temperature and moisture conditions in the atmosphere, while the processing, wrapping and sealing of many of our food products is possible on a large scale only because the air in the factories is "manufactured" to order

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[1] Lindsay D C J Indust. & Engin. Chem 21 502 1929

It is obvious that, when the air of a manufacturing plant is conditioned, the persons employed are benefited. Since happiness and efficiency are increased when people are physically comfortable, many installations of air-conditioning apparatus have been made for this purpose alone. Furthermore, uniform air conditions during working hours tend to minimize the economic and industrial losses from the common so-called respiratory diseases. The use of cleansed, cooled and dehumidified air in summer and of warmed and properly humidified air in winter cannot but add to the enjoyment of a performance in a movie theater.

While modern industry is thus increasingly availing itself of the products of scientific research, the effects on the individual employee are not always so beneficial as in the case discussed; indeed, the circumstances surrounding the manufacture of certain widely used products may constitute an industrial hazard of the most dangerous type. Only recently attention has been directed in these columns[2] to the insidious effects of the radiations from radium paint used to letter watch dials, speedometers and similar pieces of apparatus. The outlook for those affected is so hopeless that renewed emphasis should be given to the tremendous importance of enforced prophylaxis, and nothing should be left to chance or perfunctory advice. The fabric of modern society is so complex that progress made in one direction usually necessitates readjustments, not all of which are happy.

Current Comment

THE SIGNIFICANCE OF THE KAHN REACTION

Physicians are at times in a dilemma when considering the diagnostic significance of tests for syphilis. Aside from the innumerable modifications of the Wassermann test, there are in use today a considerable number of precipitation tests. The bringing together by the League of Nations Health Committee[3] of the authors of methods or of modifications and having them examine the same serums with their respective methods was an important step in assessing the value of various tests. The Kahn test evidently stood out in the four criteria for a good method, namely, practicability, specificity, sensitiveness and clear-cut reactions. Only a few years ago this test was looked on with academic interest, the belief being still prevalent that it would be impossible for any test to replace the well established Wassermann test. The wide use which the Kahn test has been attaining in this country and abroad is clearly justified in view of the superior results that it gave at Copenhagen in comparison with other internationally known tests for syphilis. Because of the importance of laboratory tests in the diagnosis and the treatment of syphilis, it was natural that the acceptance of the Kahn test by clinicians should have been slow. From now on, however, this test will undoubtedly attain still wider use.

THE PHYSIQUE OF MEN OF SCIENCE

There is a widespread impression that those persons who should be most familiar with the fundamental principles that make for human well being are all too often indifferent to their implications. Physicians who devote much of their time to the inculcation of the dictates of personal hygiene are as a class prone to neglect in their own daily lives the practices of healthful living that they insistently impose on others. Scholars in many walks of life are often assumed to be seemingly forgetful of the adage mens sana in corpore sano. The cartoonist is more than likely to represent the typical college professor as a person of frail structure who appears to struggle through life with the self-imposed handicap of a neglected body. Pasteur remarked that science should be the highest personification of nationality because, of all the nations, that one will be foremost which shall be first to progress by the labors of thought and of intellect. Such eminence is not likely, however, to be achieved by physical weaklings. Fortunately, observations that have lately been completed by Aleš Hrdlička[1] of Washington give no occasion for the traditional beliefs about defective or inferior physiques, at least so far as American scientists are concerned. He has carefully measured without choice a hundred members of the National Academy of Sciences. This represents about half of a membership rigidly selected from among the foremost representatives of the different sciences. They are, therefore, a selected group of high-class brain workers. The most striking fact brought out by Hrdlička's examination is that these men, barring rare exceptions, are in no way inferiors but rather superiors in physique, strength, health and longevity, as compared to the American and even the Old American population at large. It is decisively not a case of strong minds in weak bodies but of strong minds with strong constitutions. Furthermore—and this Hrdlička regards as especially significant—the head (and hence presumably also the brain) of the members of the academy exceeds in size not only that of the population at large but even that of the well educated and professional Old Americans. It is clear, he adds, that outside of the prevalence of above-the-average physique and above-the-average size of head, the highly talented men of science conform in their traits with the racial groups to which they belong. One may conclude with the Washington anthropologist, that in all probability, in science at least, the strongest and ablest healthy minds go generally with strong, healthy bodies, that intensive prolonged mental work is concomitant with larger-than-average head and brain, and, finally, that such intensive mental work evidently does not tend toward a shortening of the life of the workers. Perhaps this will at last dispose of the conventional belief that an eminent man of science is a person one-sided in mind, with a frail or neglected body.

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[2] Radioactivity of Bone, editorial, J. A. M. A. 92: 1447 (April 27) 1929.

[3] Report of Second Laboratory Conference on Serodiagnosis of Syphilis, Geneva, League of Nations Health Organization, 1928. See also Current News, The Journal, June 22, 1929, page 2111.

[1] Hrdlička, Aleš: Measurements of One Hundred Members of the Academy and What They Show, Science 69: 503 (May 10) 1929.

Association News

MEDICAL BROADCAST FOR THE WEEK

The American Medical Association Morning Health Talks and Evening Health Hints from Hygeia

The American Medical Association broadcasts daily at 11 15 o clock in the morning, central daylight saving time, over Station WBBM (770 kilocycles, or 389 4 meters)

The program for the week of July 8-13 will be as follows

July 8 Medical News by Dr J F Hammond
July 9 Why Fear an Anesthetic? by Miss Mildred Whitcomb
July 10 Mental Fatigue, by Miss Lois Stice.
July 11 The Girl Who Grew Up Too Soon" by Miss Mildred Whitcomb
July 12 Medical News by Dr J F Hammond
July 13 Hunger and Thirst by Mr Cecil Bean

Evening Health Hints from Hygeia, 8 o'Clock, Central Daylight Saving Time

July 8 Skimmed Sunshine.
July 9 Purifying Cannery Waste
July 10 Economic Factors Help Bring on Tuberculosis
July 11 Garlic Scents Breath for Seventy Two Hours.
July 12 Shyness is a Mental Disease
July 13 College Students Must Learn How to Eat

Medical News

(Physicians will confer a favor by sending for this department items of news of more or less general interest such as relate to society activities new hospitals education public health etc)

CALIFORNIA

Chiropractor Given Life Sentence —Arthur O Berg a chiropractor of Los Angeles, after serving eight months in San Quentin Penitentiary, returned to Los Angeles in May for a new trial, the decision of the lower court having been reversed Berg pleaded guilty to a charge of manslaughter as a result of criminal abortion which resulted in the death of a 15 year old university student He withdrew his plea of guilty but was convicted at the second trial and sentenced to life imprisonment at San Quentin (The Journal, Sept 15, 1928 p 806)

Dr Bolt Goes to Western Reserve —Dr Richard A Bolt, assistant professor of child hygiene and lecturer in pediatrics University of California, has resigned to accept an appointment as director of the Cleveland Child Health Association associate professor of hygiene and public health, Western Reserve University Cleveland, and associate in preventive pediatrics at the medical school Dr Bolt has been at the University of California since 1925 He is a graduate of the University of Michigan Medical School and of Johns Hopkins University School of Public Health

Society News —The Northern California Medical Golf Association held a tournament at Sonoma Mission Inn, June 15 Suggestions as to future tournaments should be made to Dr Harry E Alderson, 490 Post Street, San Francisco ——The National Research Council has given about $1,500 to the University of California for sex research to be carried on by Dr Herbert M Evans ——The attendance at the recent state medical meeting at Coronado exceeded any previous registration by 302 ——Six lepers from California were sent to the national leper home at Carville La May 15 Four of them were from San Francisco one from Oakland and one from Los Angeles Since 1922 California has sent eighty eight lepers there, nearly all of whom were Mexicans and Orientals

Leprosy Organism in Man and Rat —In addressing the Western Society of Naturalists at Berkeley Ernest L. Walker, S D professor of tropical medicine University of California Medical School, expressed the conclusion that the organism causing leprosy in man is identical with the organism causing leprosy in rats It belongs, he said among *Actinomyces* Since the leprosy bacillus was discovered in 1871 by Hansen, its artificial cultivation has been unsatisfactory, producing usually forms having apparently no relation to the bacillus found in the lesions The organism is not a simple bacterium Dr Walker said, but it assumes a variety of forms during its life history He believes that the actinomyces of leprosy is a saprophyte which lives in the soil and only by chance becomes a parasite on gaining admission into man Stefansky in 1903 found that a disease exists in rats closely similar to leprosy in man Dr Walker said they have been able to cultivate the rat organism and have found it to be an actinomyces They inject rats regularly with these cultures and have recovered the organism after more than a year Although their series of rats amount to hundreds a case of cutaneous lesions has not yet been produced Their research, he believes, will throw light on human leprosy and provide a means of proving the etiologic relation of the soil actinomyces to the disease

Personal —Dr Jacob Schwarz, San Francisco, is reported to have been willed $6,500 by the late Helene Brock, aged 70, of New York Dr Schwarz attended Miss Brock when she was ill in San Francisco in 1925 ——Dr Rupert G Doupe has been appointed health officer of the city of Tehachapi to succeed Dr Clara M S Rinehart ——Dr Ray Lyman Wilbur, Secretary of the Interior gave the commencement address at Stanford University, June 17 1 071 degrees were conferred the class being the largest in the history of the university ——Karl F Meyer, Ph D , professor of bacteriology and director Hooper Foundation for Medical Research, University of California, has gone to Europe to study malta fever and public health problems in the canning industry ——Robert G Sproul vice president and comptroller of the University of California, has been made president of the university, effective July 1, 1929, on the retirement of Pres William W Campbell, LL D Dr Sproul is 38 years of age, a native of California, a graduate of the university in civil engineering and has the degree of LL D conferred by Occidental College.——Dr Edgar Van Norman Emery has resigned as director of the Los Angeles Child Guidance Clinic to accept a position at the newly organized Institute of Human Relations at Yale University, New Haven, Conn ——John F Kessel has been appointed associate professor of bacteriology and parasitology at the University of Southern California School of Medicine.

How Many Physicians Are Licensed?—The new edition of the American Medical Directory which has just been completed, contains the names of 168 201 physicians who are licensed in the United States and its possessions and Canada In this number are about 12,000 names which did not appear in the edition published two years ago The actual increase in names over the preceding edition of the directory is, however, only about 4,199, because of the fact that nearly 7,000 have died since the tenth edition was published

GEORGIA

Changes in Medical Faculty —The president of the board of trustees of the University of Georgia has announced the appointment of Dr James E Rush as head of the department of public health, effective at the beginning of the next school year, and of Dr Erich W Schwartze as head of the department of physiology, succeeding Dr William Salant resigned Dr Rush is doing graduate work at Yale University previously he was professor of public health at the University of Kentucky, Lexington Dr Schwartze comes to Augusta from the University of Pittsburgh

Society News —The Fulton County Medical Society was addressed June 20 by Drs Hal C. Miller on Pyloric Obstruction Following Appendectomy ' Madison Hines Roberts, on Breast Milk,' and William L Champion and Major F Fowler on Gonorrheal Arthritis' ——Dr Charles T Nellans has resigned as chief physician at the United States Federal Penitentiary Atlanta to devote his time to private practice ——The Fulton County Medical Society was addressed June 6 by Dr Charles W Roberts on differential diagnosis of acute appendicitis, Dr John B Fitts New Ideas of Food and Nutrition" and Dr Frank K. Boland, mortality of acute intestinal obstruction

Tuberculosis Facilities Inadequate —The tuberculosis situation of Atlanta and Fulton County has been improving for years but the facilities are considered still inadequate The *Bulletin* of the Fulton County Medical Society says there is a waiting list at Battle Hill of more than 200 and at Alto of about 150 and it requires months to get a patient into these institutions The tuberculosis death rate in Fulton County in 1928 was 146 2 per hundred thousand of population The local tuberculosis association believes Atlanta should have a nursing home where patients could be sent to have the advantage of a sanatorium and at the same time be treated by their own family physician if they desire Good work is being done at present among the children and it is believed that the general tuberculosis situation will continue to improve. The cooperation of the practicing physicians of the county is requested

ILLINOIS

Community Celebrates General Practitioner's Birthday—Hundreds of friends gathered in the gymnasium of the high school at Mount Zion June 9 for a banquet to celebrate the seventieth birthday of Dr Sheffie R. May, who has practiced there about forty-two years This appreciation of Dr May's service to this community was sponsored by the Commercial Club and was attended by friends from outlying districts where the guest of honor had often gone to serve them Dr May was presented with a watch and chain, and Mrs May with a diamond brooch The number present may be estimated from the fact that 35 gallons of ice cream was served

Personal—Dr Edward S Murphy, Dixon, has been elected president of the Alumni Association of Rush Medical College for the ensuing year——Dr Herman L Horwitz has resigned his position as assistant medical director of the Cook County Tuberculosis Hospital, Oak Forest, effective July 5——After nearly ten years' service as health commissioner of the city of Rock Island Dr Albert N Mueller, has resigned and announced his retirement from the general practice of medicine, in which he has been engaged for about twenty-two years His successor will be Dr Ralph Dart, who was health commissioner previous to the time that Dr Mueller was appointed. Dr Dart has been practicing in Rock Island since 1904

Chicago

Personal—Dr Otto F Kampmeier has been appointed head of the department of anatomy of the University of Illinois College of Medicine to succeed the late Prof Victor E. Emmel——Dr Zoia Tschoumacova, recently of Chicago and formerly in practice in San Francisco, has accepted a position on the medical staff of the Northern Colony and Training School at Chippewa Falls Wis——Dr Robert A. Black, professor of pediatrics, Loyola University of Medicine, will have charge of the new $300,000 La Rabida Sanatorium in Jackson Park, which will soon be under construction The sanatorium will care for children who have heart disease——Dr Egil T Olsen, who resigned as superintendent of the Englewood Hospital about four months ago has been appointed superintendent of the Receiving Hospital Detroit

From Gongs to Sirens—The Chicago School of Sanitary Instruction whose bulletin is edited by the city health commissioner, devotes a recent issue to noise as a menace to health Tests made on normal subjects it is said, showed that 19 per cent more energy was required to perform certain duties in noisy locations than was required to perform them in quiet locations Noise that is not sufficient to wake a sleeping person will cause his muscles to remain tense as long as thirty minutes Sleep under such conditions is not refreshing Sanitary engineers have made tests in Chicago by means of the audiometer an instrument which determines the volume of noise in any locality on a percentage basis The continuous sound or community noise which they found in various localities varied from 8 to 10 per cent in the country to 15 per cent in the suburbs from 25 per cent in residential districts to 35 per cent in industrial districts and up to 40 per cent in the loop The increase in noise in Chicago is further shown by the fact that it has become necessary for the fire and police departments to use sirens, while five years ago they used gongs Noise is now a serious problem. Several hospitals are considering moving to quieter areas and the number of protests received by city authorities against noises is increasing each year The Chicago School of Sanitary Instruction is conducting studies with a view of presenting details to the legislature for the enactment of laws which will aid in controlling unnecessary noise

KENTUCKY

Society News—Dr James Vernon Pace, Paducah has been elected president of the Southwestern Kentucky Medical Association for the ensuing year——The Bourbon County Medical Society Paris was addressed May 9, on 'Toxemias of Pregnancy by Dr Scott D Breckinridge, Lexington——Dr William F King Indianapolis secretary Indiana State Board of Health, addressed the Fountain-Warren County Medical Society Covington June 6 on Undulant or Malta Fever' This was the annual Wabash River catfish dinner meeting——Dr Clarence J Broeman Cincinnati addressed the Scott County Medical Society Georgetown, June 6 on 'Treatment of Cancer of the Fundus——The model of the statue of Dr Ephraim McDowell, which was placed in the Capitol Building at Washington a few months ago, has been procured by the Kentucky State Medical Association, and will be installed in the capitol at Frankfort——Dr Gordon B Underwood, Evansville, Ind, conducted a skin clinic before the Daviess County Medical Society, Owensboro, June 11, and addressed the society at a banquet

MASSACHUSETTS

Esculapius' Family on the Stage—A play entitled "Esculapius," written by Dr Barbara Ring of Arlington Heights, was staged, June 23, on the occasion of the fiftieth anniversary of the Ring Sanatorium and Hospital The time was 1329 B C, the place, Epidaurus, Greece, and the setting the Temple of Esculapius, dedicated to Apollo Among the characters were the daughters, Panacea and Hygeia, the mother Epione, and the sons, Machaon and Podalirius Apollo's part was played by the well known actor Alan Hamilton The play depicts the practice of medicine in ancient times and portrays the ideal physician Esculapius, on whom the god of the lower world reaped vengeance for restoring the sick and thus withholding their shades Apollo then comes to earth and raises Esculapius to the realms of the gods and dedicates to his followers his staff, the caduceus, which remains to this day the symbol of the profession The principal parts in this presentation were taken by Boston professional and amateur players who were assisted by the choruses from the Arlington and Belmont women's clubs It was presented in the open garden of the Ring Sanatorium before an audience of 1,200

MINNESOTA

Personal—Dr William J Mayo, Rochester, received the honorary degree of doctor of science from Marquette University, Milwaukee, June 12, on which occasion he delivered the commencement address——Dr Fredolph H Magney has been elected chief of staff of St. Luke's Hospital, Duluth for the ensuing year——Dr Charles H Mayo, Rochester, will address the British Medical Association at Manchester in July on "The Mechanism of Abdominal Pain"

Club Proposes Postage Stamp to Commemorate Morton—At a meeting of the Exchange Club of Duluth June 10, a resolution was adopted requesting that the U S Post Office Department commemorate the name and achievement in the forthcoming new series of postage stamps of William Thomas Green Morton, Boston, who, the resolution says, "discovered and promulgated the anesthetic effects produced by the inhalation of sulphuric ether"

Society News—The Southern Minnesota Medical Association spent the afternoon of June 15 on the Mississippi River near Winona at the dinner Dr Arthur R Metz, Chicago, presented motion pictures of big game hunting in Africa Other speakers were Drs Hans M Lichtenstein, Winona, on injection treatment of varicose veins, and Everett K. Geer, St Paul, on iodized oil in the diagnosis of thoracic conditions Clinics were held by Drs James T Christison and Ernest M Hammes, both of St Paul, and by Dr Owen H Wangensteen, Minneapolis

Horsley Prize Awarded to Dr Horton—The John Horsley Memorial Prize for 1929 has been awarded to Dr Bayard T Horton of Rochester, for a thesis entitled "A Study of the Pyloric Block with Special Reference to Musculature, Myenteric Plexus and Lymphatics" The prize, given by Dr John Shelton Horsley of Richmond, Va is awarded biennially by a committee of the University of Virginia Faculty to medical graduates of the University of Virginia of not more than fifteen years' standing for a thesis representing original work on some surgical subject The prize consists of the income for two years from a gift of $10,000 and amounts, therefore to about $1 000 Theses in competition for the next award must be received by the chairman of the committee, Dr Edwin P Lehman, University, Va, by Feb 1, 1931

NEW HAMPSHIRE

Another Kind of Behavior Clinic—The first behavior clinic on the mental hygiene program of the state board of health was held at Durham, June 5 in connection with a regular conference for babies and children of preschool age conducted by the maternity, infancy and child hygiene division Sixty-six children attended the conference This new work is to assist parents to avoid bad mental habits in children After the children were physically examined, they were passed on to the psychologist The state board says that almost without exception parents approved this work and that most of them had problems which they wished to discuss with the psychologist It is planned to hold these clinics throughout the state Follow up work will be done by nurses The physical examinations at the first clinic were conducted by Drs Albert E. Grant, Durham, and Franklin N Rogers Manchester, and the behavior clinic by Dr Anna E C Rudd Durham, and Mrs E. A Phillips, psychologist to the University Nursing School

NEW YORK

Society News —The Medical Society of the County of Albany was addressed, June 26 by Dr Philip L Forster, Albany, on "The Orr Treatment of Osteomyelitis '——The Fulton Academy of Medicine has appointed a committee to inaugurate a memorial day for deceased physicians The emblem for marking their graves will be the caduceus

Facing an Enormous Mental Problem.—In seven years the people of New York have approved bond issues totaling $150,000,000 to provide care for their people who are mentally ill Now the governor is urging the legislature to permit another $50 000,000 bond issue to go before the people this fall There is said to be one mentally ill person in every five families in the state. During 1928, there were under treatment in the fourteen civil state hospitals for the insane, 58,354 patients in addition to the 9 124 in the four state schools for mental defectives and the 1,890 in the institute for epileptics Overcrowding in some of these hospitals is said to be shocking amounting on April 1, to 10 625 While the state is building the largest hospital for mental patients in the world at Brentwood in Suffolk County (eighty buildings will eventually be erected) several years will elapse before this entire facility will be available This hospital, which was named in honor of Dr Charles W Pilgrim, will cost about $30 000,000 and accommodate 10 000 patients Governor Roosevelt has considered calling a special session of the legislature to provide for another bond issue, and a committee of the legislature has been inspecting the state hospitals The committee seems to hold the opinion that sufficient funds have already been made available by the legislature through the two bond issues and appropriations from current revenue to house properly and care for the patients The committee believes there has been an unwarranted delay in the state architect's office in carrying forward new construction It pointed out instances wherein four years had elapsed after the money was provided before the beds became available

New York City

Personal —A portrait of Dr Max Rosenthal dean of the staff of Sydenham Hospital, was presented to the institution June 13 ——Dr Haven Emerson, professor of public health administration, Columbia University College of Physicians and surgeons gave a series of addresses June 5, under the auspices of the Mahoning County Medical Society Youngstown Ohio ——Dr James J Walsh, who has been reelected president of the Catholic Writers Guild of America guest of honor and principal speaker at the annual banquet of St Louis University, St. Louis, June 3

Oldest Hospital Has a Deficit.—The New York Hospital, whose charter was granted in 1771 by King George III of England, recently celebrated its one hundred and fifty seventh anniversary The annual report showed that during 1928 the hospital operated at a deficit of $163,264 About 45 per cent of its ward service was given free to needy patients and about 40 per cent of its outpatient service The New York Hospital in cooperation with Cornell University, has just started construction on a great medical center on the East River, to which it will move when the buildings are finished

Health Department Hopes to Abandon Clinics —In conducting the campaign to eliminate diphtheria the city health department has found that parents who would prefer to pay a small fee to have their children immunized by a private physician take them to one of the special stations maintained by the department because they fear that the physician s fee might be too high The Diphtheria Prevention Commission with the cooperation of the New York County Medical Society will circularize physicians in Manhattan to ask whether they will set aside certain hours to give toxin antitoxin to children for $2 an injection The health department prefers to have these immunizations done by private physicians It hopes the profession will assume this and other similar responsibilities and thus make possible the gradual abandonment of many of the clinics health stations and other activities which the department feels it has been compelled to establish to meet the present situation.

Fraudulent Health Inspector Bibeau Imprisoned —For several months Joseph Bibeau an imposter went about representing himself as an inspector of the New York City Department of Health victimizing restaurateurs barber shops and beauty parlors by collecting $10 for the renewal of their permits When first arrested sometime ago on Staten Island Bibeau was released on bail and he immediately resumed his illegal operations in other parts of town He was rearrested in May and found guilty and was sentenced, June 3 to the workhouse for an indeterminate sentence which means, it is said from six months to three years Bibeau s record showed that he had served prior jail sentences A letter from the Ohio State Medical Board in 1920 stated that one Joseph Percy Bibeau was licensed to practice electrotherapy and was then located in Cleveland. There is also on file a clipping from the Cleveland *Plain Dealer* in 1914 of an advertisement of 'Dr J P Bibeau 229 Colonial Arcade to remedy flat noses, hollow cheeks and falling hair This Mr Bibeau was born in France

Quack Goldenberg Convicted —Salvi Goldenberg who dubs himself "Professor," was convicted in Special Sessions Court New York County, May 26 1929, of practicing medicine illegally According to reports, Goldenberg came to New York from Vienna five years ago and began to practice physical therapy As the law requires that such practice shall be at least under the supervision of a licensed physician, Goldenberg seems to have obtained the help of Dr Joseph P Essner and later, Dr J Marino Goldenberg told a woman who went to his office that she was suffering from gallstones and is said to have offered her a preparation, which he guaranteed would cure her, at $2 a bottle. It was necessary for the patient to take twenty-four bottles When the 'medicine was tested by the chemists of the health department it was found to consist of 99 4 parts of water and no medicinal agents Part of the evidence presented in the Goldenberg case was a series of contracts written in German in which were promises to cure gallstones, diabetes and other ailments

Who Is Practicing Abroad?—The eleventh edition of the American Medical Directory, which is now ready to ship, contains, for the first time, a list of American physicians who are located in foreign countries including those engaged in research or graduate study as well as medical missionaries and those employed by industries or otherwise temporarily absent. The list is on page 1689 The directory is published by the American Medical Association, 535 North Dearborn Street, Chicago

OREGON

Personal —Dr Randall F White Portland, has been appointed medical director of the Russian Caucasus for the Near East Relief He will have charge of a children s hospital at Leninakhan in southwest Russia, and of traveling clinics in adjacent provinces He and his family sailed last month

Dr McCool Goes to California.—The University of California Medical School announces that Dr Joseph L McCool has accepted an appointment as associate clinical professor of ophthalmology effective July 1 Dr McCool for more than twenty years has practiced in Philadelphia and Portland, and has taught at Temple University School of Medicine University of Pennsylvania Post Graduate School of Medicine and University of Oregon Medical School He is a past president of the Pacific Coast Oto Ophthalmological Society

Hospital News —Since the U S veterans bureau hospital opened in Portland, the turn-over of patients has been about 350 a month, a rate which tests the capacity of the hospital The new hospital is said to be equipped with every facility for the care of general medical and surgical cases and to have one building for the care of psychotic patients and another for tuberculous patients The hospital is near the medical school of the University of Oregon, the county hospital and the children s hospital It has had the hearty cooperation of local physicians

PENNSYLVANIA

Philadelphia

Personal —Dr Temple S Fay has been made head of the department of neurosurgery at the Jewish Hospital Philadelphia ——Dr G R Anderson has been elected president of the board of trustees of the Spangler Hospital ——Many of the delegates to the International Hospital Congress at Atlantic City from foreign countries were guests of the Philadelphia Hospital Association at a dinner June 12 Dr Joseph C Doane a past president of the American Hospital Association was toastmaster ——Dr P Brooke Bland has been appointed visiting physician to the Preston Retreat for the ensuing year

University News.—During the commencement exercises at the Woman s Medical College of Pennsylvania ground was broken for the first units of the $1 000 000 building on the new site at Henry Street and Abbottsford Road, Falls of Schuylkill Eighteen women were graduated from the college June 12 The alumni dinner was attended by sixty women physicians Dr Martha G K. Schetky, president of the alumni association, presided——At the annual meeting of the medical alumni of the University of Pennsylvania, June 15, Dr Charles K. Mills, the oldest alumnus present, was presented with a cup, as were Drs Henry W Himmelberger, Reading, and Gordon T Atkinson, Crisfield, Md., of the class of 1869 The class of '94 had the largest number of members present. Dr Edwin S Potter, '91, from Santa Barbara, Calif, was awarded the prize for coming the greatest distance Addresses were made by Drs George E de Schweinitz, Alfred Stengel and Samuel M Hamill Dr George M Coates was elected president of the medical alumni for the next year

Growth of Radiation Department—The radiologic department of the Philadelphia General Hospital has since 1922 expanded until the entire first and fifth floors of the new building have been assigned to it On the top floor eighty-eight ward beds are reserved for cancer patients, on the first floor are the clinic and the reception room, the radium treatment compartments, a workroom for making up radium applicators and packs, and roentgen-ray machines Of more than 6,000 patients who had received radium or roentgen treatments, 39 per cent were still living, although many were admitted in a feeble state The roentgen-ray department operates continuously from 9 to 5 o clock, and special night nurses are on duty to adjust, remove or reapply radium packs There are facilities at the hospital and personnel to care for any kind of cancerous lesion as well as other ailments in which radiation treatment is indicated The department hopes that the medical profession will avail itself of this service and cooperate in giving these cases the advantage of early treatment There are now among those that come an "appalling" number that are hopelessly inoperable.

TENNESSEE

Malaria Tests on Thousands of Children.—At a conference in Memphis, June 10, attended by Dr Eugene L Bishop, state health officer, Dr William B Harrison of the county board of health, Dr Lloyd M Graves of the city board of health, and Dr L L Williams of the U S Public Health Service, it was announced that a malaria index would be carried out in Shelby County on about 20 000 school children as part of a state-wide drive against malaria The object of the test would be to determine just how much malaria there is in Shelby County Many physicians and nurses will assist in this work

Survey of Thyroid Enlargements—School children in forty localities in Tennessee have been surveyed by the U S Public Health Service to determine the prevalence of thyroid enlargement Among 9,073 white and 1,739 colored boys, and 11,120 white and 3,196 colored girls there were found 4 876 thyroid enlargements, representing a percentage of 19 4 Thyroid enlargements, the public health service says, prevailed among the white boys to the extent of 9 5 per cent and among the white girls to the extent of 23 5 per cent. Among the colored boys, 84 6 per cent of the thyroids were normal and among the colored girls 64 5 per cent. All degrees of enlargement were noted For example, among the white boys, 7 7 per cent were considered very slightly enlarged 1 5 per cent as slightly enlarged, 0.2 per cent as adenomatous, and 0 04 as moderate enlargements The finding that endemic goiter is more frequent among the colored children of Tennessee may be due to nutritional and economic factors Endemic goiter is more prevalent in eastern than in western Tennessee No relationship was found between the amount of goiter and the sources, treatment and ultimate safety of public water supplies There was, however, a slightly larger incidence of endemic goiter among the users of chlorinated drinking water than among the users of unchlorinated water In Oregon the incidence of endemic goiter is said to be slightly greater among the users of unchlorinated water In surveys like this one made by the U S Public Health Service in six states and one city, the order of goiter incidence was as follows Minnesota, Cincinnati (colored) Cincinnati (white) Oregon, Tennessee (colored), Tennessee (white), Massachusetts and Connecticut

WEST VIRGINIA

Society News—The Fayette County Medical Society, Montgomery, was addressed, June 11 by Dr Thomas G Reed Elkridge on Traumatic Shock and by Dr William R. Laird Jr, Montgomery on 'The Rupture of Gastric and Duodenal Ulcers

Personal—Dr Guy Hinsdale who recently moved to White Sulphur Springs is a representative for the International Society of Medical Hydrology——Dr William H McLain has been appointed health commissioner of the city of Wheeling for a term of four years

WISCONSIN

Personal—Dr William W Morrison has been elected health officer of the city of Edgerton——Dr Edward Blumer, having been recently reelected, is now serving his nineteenth year as village president of Monticello

Society News—The Canti Cancer Film was presented before a joint meeting of the Milwaukee County Radiological Society and the Milwaukee Academy of Medicine, June 11 An address was given by Dr Stanley J Seeger on "Sedimentation Reaction in Children"——The Eau Claire and Associated Counties Medical Society was addressed recently by Dr William C Carroll, St Paul, on "Cervical Ribs"——The Jefferson County Medical Society was addressed, in April by Dr Chester M Kurtz, Madison, on "Determination of Cardiac Enlargement and Its Significance in Diagnosis"——The Marathon County Medical Society, Wausau, presented, May 1, three reels of motion pictures on "Diagnosis and Treatment of Syphilis and Gonorrhea"——The Marinette-Florence County Medical Society gave a dinner in honor of Dr and Mrs Edward Sawbridge of Stephenson, Mich, whom they presented with a silver service——Dr Lyman A. Copps Marshfield addressed the annual meeting of the Ninth Councilor District Medical Society, Stevens Point, on 'Effects of Increased Intracranial Pressure , Dr Carl W Eberbach, Milwaukee, on "Medical Experiences Among the Labrador Eskimos," and Dr Joseph F Smith, Wausau on "Intestinal Obstruction"

GENERAL

Bath Tubs Less Popular Than Cars—A survey made by the General Federation of Women's Clubs in 1925 indicated that only 68 per cent of city homes were equipped with stationary bath tubs According to the Illinois state health department, from 1913 to 1922, enameled bath tubs were installed at the rate of 480,000 a year in the United States This figure increased to an average of 1,100,000 a year from 1922 to 1928 Bath tubs still appear to be less in public favor than automobiles

Deaths by Violence Increase—The National Safety Council, with headquarters in Chicago, has published the results of the annual survey showing that 96,000 persons met accidental death during 1928 in the United States, an increase of 3 1 per cent over the previous year The council reports that 27,500 persons were killed in automobile accidents, or 6 3 per cent more than in the preceding year Railroad and street car accidents decreased Aviation is said to have taken 368 lives, among whom those of passengers exceeded those of pilots There were said to be 24,000 accidents in American homes The home tragedies, as the result of fires and explosions, numbered 6,000, those due to asphyxiation and suffocation, 4,000, those due to poisoning, 2,000, and the remainder, or about 40 per cent, were due to such accidents as falls on slippery floors and rugs

Health Officers of North America—The forty fourth annual meeting of State and Provincial Health Authorities of North America, Washington, D C., June 1, was addressed, among others, by Dr Aquilino Villanueva, chief health officer of Mexico Dr Frederick E Trotter, health officer of the Territory of Hawaii, was elected president for the ensuing year, and Dr Eugene L Bishop, health officer of Tennessee, vice president, Dr A J Chesley, St Paul, executive officer of the Minnesota State Board of Health continues as secretary The organization adopted a resolution urging passage of the Capper-Bankhead bill to provide for the development of an organization throughout the country for rural sanitation The organization also went on record as favoring the passage of the Jones Cooper bill to continue in operation the division of maternal and child health in the U S Department of Labor

Disbursements by Rosenwald Foundation.—The latest gifts announced by the Julius Rosenwald Foundation, Chicago amounting to $205 000, are to aid hospitals in the south and east to maintain negro public health nurses in fourteen southern states, and for a negro health survey The hospitals awarded funds on condition that the community raise funds also are the Charity Hospital, Savannah Ga, $50,000 toward a new building, St. Agnes Hospital, Raleigh, N C, $15,000 toward a nurses home and outpatient service Richardson Memorial Hospital, Greensboro N C $15 000 toward a nurses' home, Mercy Hospital Philadelphia, $35 000 and Provident Hospital, Baltimore, $60,000 The foundation appropriated $30 000 for a negro health survey to be made by the Tennessee State Health Department cooperating with the U S Public Health Service and Fisk University fond of old scientific books as to spend his holidays browsing through the book shops in England, endeavoring especially to obtain fundamental contributions He acquired many books published in the fifteenth century The library which Osler gave to McGill University contains 7,600 volumes selected with a view to their value in the study of history of medicine and science These were by no means all of the books which filled his house at Oxford. He left to Johns Hopkins Hospital a collection of works on the heart and lungs, and gave to the Tudor and Stuart Club of Johns Hopkins University, which he and Lady Osler founded in memory of their son, another collection. McGill University has provided a room for Osler's books, arranging them on shelves as nearly as possible in the catalogue order His own writings are arranged at the end of the room in the alcove on one side and on the other are those of his favorite authors Behind a panel bearing his portrait, as he desired, rest his remains Four portraits hang in the room, one of Osler made in 1885 shortly after he left McGill, the others are of Harvey, of Sydenham and of Linacre, the translator of Galen

PHILIPPINE ISLANDS

Newspapers Play Up a "Discovery"—In some manner, the newspapers in Manila obtained and published an item, recently, announcing that two local physicians had discovered a cure for typhoid The editor of the *Journal of the Philippine Islands Medical Association* says that the press not only played up the item but lauded it editorially creating no little commotion The wisdom of such an announcement is not clear, in view of the fact that the "discoverers" in this case refused to give out the formula The Manila show is over, and a remedy has been suggested for future impresarios of this sort The Filipino law provides that any certificate of registration may be revoked on unprofessional conduct which involves advertising by any person, firm, association or corporation in a written or printed paper in which untruthful or improbable promises are made Medical ethics is as necessary in Manila as it is in Chicago or elsewhere

FOREIGN

Physicians in Parliament.—As a result of the recent general election in Great Britain, there are now eighteen registered medical practitioners in the new House of Commons as against thirteen in the old Two physicians—Lord Dawson and Lord Moynihan—are members of the House of Lords The new minister of health is Mr Arthur Greenwood, and his parliamentary secretary Miss Susan Lawrence Mr Greenwood has had experience in the ministry, having been parliamentary secretary in 1924

American Public Gives Hospital to Japanese —The Fraternity Hospital at Tokyo, one of the best in Japan, was opened, June 1, on the site in a populous section where thousands of people perished in the earthquake of 1923 The $1,500,000 building is a modern American steel and concrete structure accommodating 248 patients, with an outpatient department accommodating 600 The entire cost of construction and the $2 000 000 reserve fund which it has are the last remnant of the great outpouring of sympathy which the American people gave at the time of the disaster and forwarded to Japan as the American Relief Fund Prince Tokugawa at the opening ceremony said that the hospital will serve not only the sick but as an eternal memorial to the friendship of Japan and America and as an everlasting symbol of human brotherhood'

British Medical Prizes—The British Medical Association announces that it will consider the award of the Katherine Bishop Harman Prize in 1930 (about $400), which is intended to encourage study directed to the diminution of risks that are apt to rise in pregnancy and childbearing Any practitioner in the British empire is eligible to compete. The Sir Charles Hastings Prize, which is open for award in 1930 was established to promote observation research and record in general practice Its money value is 50 guineas The association will award in March 1930, prizes of about $125 for the best essays on Three Cases Illustrative of the Value of Antenatal Observation of Pregnant Women' by final year medical students or by registered practitioners of not more than one year s standing The association is prepared to receive applications for the Ernest Hart Memorial Scholarship of $1 000 per annum and three research scholarships each of about $750 per annum These will be given to candidates whom the science committee of the association recommends as qualified to undertake research in any subject (including state medicine) relating to the cause, prevention and treatment of disease The address of the British Medical Association is Tavistock Square, London, W C. 1

American Hospital in Constantinople Needs Funds—Philanthropic Americans averted the closing of the American Hospital of Constantinople, recently, by donating about $16,000 to meet expenses The hospital was forced to move and to renovate another building, the lease on the old building having expired It still needs financial assistance Established in 1920, chiefly to provide care for Americans, it has had assistance from the American Red Cross, Near East Relief and American business men The American colony in Constantinople at times numbers several hundred The hospital served a special need during the turbulent situation following the Great War, in caring for many refugees An important part of its work has been the training of nurses, eleven graduating in March, 1928, and six in January, 1929 American standards have been maintained in training nurses, although they are mostly Russians, Belgians and Armenians An arrangement has been made with American Women's Hospitals, New York, whereby it will support six beds for children in the American Hospital of Constantinople Two great needs of the hospital are a building of its own, and some organization in America which would assume the task of supplying funds to meet the annual deficit The medical director is Dr Lorrin A Shepard, a graduate of Columbia University College of Physicians and Surgeons, New York. A board of American business men and others attends closely to the administration (The Journal, April 21, 1928, p 1302)

Government Services

Examination for Entrance to Public Health Service

Surgeon General Cumming announces that an examination will be held September 9, at Washington, D C., Chicago, New Orleans and San Francisco, for entrance into the regular corps of the U S Public Health Service. Requests for permission to take the examination should be addressed to the surgeon general, U S Public Health Service, Washington, D C Candidates must be 23 and not over 32 years of age, graduates of a reputable medical college, and must have had one year's hospital experience or two years' professional practice The examination will consist of oral, written and clinical tests before a board of officers, and a physical examination. Those who pass will be recommended for appointment by the President with the advice and consent of the Senate

Army Personals

Col Robert M Blanchard is detailed as an instructor of the Georgia National Guard with station at Atlanta on completion of his present tour of foreign service.——Capt Charles C Gans, Fort McPherson, Georgia, will proceed to his home about July 15 and await retirement at the convenience of the government ——Major Jacob C Bowman will sail from San Francisco about October 5 for New York, proceeding from there to Fort Monroe, Va for duty and on his arrival Capt Everard Blackshear will stand relieved from duty and proceed to Fort Moultrie, South Carolina ——Major Daniel R Chase will sail from New York about October 14 for San Francisco and then proceed to Fort Huachuca, Arizona ——Capt. Walter F Tolson was to proceed from the general depot, Brooklyn, about July 5 for the Walter Reed General Hospital for duty ——Capt Hubert S Steenberg has been retired from active duty, having been found incapacitated on account of disability incident to service

Navy Personals

Lieut. Cyrus C Brown has been transferred from Guam to San Diego, Calif Lieut. Comdr Wilbur O Manning from Haiti to Philadelphia, and Lieut Charles G McCormack from the Virgin Islands to Hampton Roads, Va Lieut Lester E McDonald has been transferred from Mare Island, California to a destroyer squadron of the battle fleet and Lieut Edward M Steger from the fleet to San Diego Calif Lieut Oscar W Thoeny has resigned from the navy effective July 16 Comdr Harry R. Hermesch has been transferred from the navy yard, Washington, D C, to the U S S *Tennessee* and Lieut. Charles Wheatley to the bureau of medicine and surgery Lieut William M Cason resigned from the navy, effective July 1 Lieut. Bernard S Pupek has been transferred from San Francisco to the navy hospital, Boston, and Lieut. William C Baty, Jr, to the navy hospital, Washington, D C

Foreign Letters

LONDON

(From Our Regular Correspondent)

June 8, 1929

War Pensions

The largest single vote for any of the great departments in the house of commons was $268 000,000 for the ministry of pensions The number of beneficiaries is about 1 500 000 At the end of the current year no less than $4 565 000 000 will have been spent on war pensions, compared with a little more than half that sum by Germany and less than half by France The reason for the difference is that the rates in this country are much higher A 50 per cent disabled man get $5 a week as compared with $115 a year in France and $90 in Germany This year's estimate shows a decrease of nearly $17,500 000 but for the next ten years the mean expediture will be about $180,000 000 The item that has contributed most to the decline this year is the reduction in the number of children to whom allowances were given The demand for medical treatment is declining and the hospital accommodations of the ministry are reduced accordingly The ministry still retains seventeen hospitals with about 3 000 beds and has a call on other hospitals with accommodations for 2,000 cases The number of patients in the hospitals is 5,106

The Spread of the Smoke of Towns into the Country

Prof J B Cohen, addressing members of the Leeds Luncheon Club, said that a phenomenon he had noticed recently proved definitely that smoke from industrial centers spread for many miles into the country A rain cloud would absorb soot and smoke and almost become saturated with it, just as fog would absorb it When that rain cloud, saturated with soot and tar, fell as rain the soot was liberated and came down with the rain On the previous day, near Coniston it was drizzling all day, and he noticed that the surface of the lake became coated with a thin film of greasy matter It was significant that the wind was blowing direct from Yorkshire As the wind blew the film toward him, the water near the shore became coated with a black fringe of soot He took out a piece of rag and placed it on the surface of the water In evidence, Professor Cohen held up a piece of rag which was black all over with thick, greasy soot That, he said came from Yorkshire Since the war a good deal had been done in the factory, and the boiler chimney producing smoke would one day be obsolete, but 40,000,000 tons of coal was burned annually in domestic fireplaces Six per cent at least went up the chimney as soot That was to say, 2 400 000 tons of soot fell over the land, and of that quantity a fourth was tar The increasing discoloration of trees and shrubs was due not to factories but to domestic soot.

Compulsory Health

Dr William G Savage, health officer for Somerset in an article issued under the auspices of the Central Council for Health Education, advocates the adoption of 'compulsory health" By that he means the duty of all individuals in the community to maintain themselves in health or if they are in charge of children, to see that they are maintained in health, all, of course, so far as is practicable—a duty if necessary to be enforced by compulsion. In the old days when one did not care what happened to one's neighbor such an idea would have seemed absurd, but in many ways people are advancing steadily toward an acceptation of it. It is logical to demand that members of the community shall not deliberately live under unhealthful conditions that will predispose them to develop tuberculosis and if infection is also added largely insure their acquiring it Those penalized by such neglect include a far wider range than the sufferer himself and involve expenditure for the community which need not have been incurred If it is right for the community to expend large sums of money on maintaining and assisting its members to maintain or to regain health it is but a logical sequence that its members must themselves play their part in maintaining their own health Any neglect on their part is antisocial and will in time be recognized as such Before however, any such demand can be made on members of the community they should have the opportunity of knowing much more about the laws of health and how health can be maintained There is urgent need for an extensive system of general instruction on health matters and Savage believes that in a few years this will be recognized as an essential part of the work of every health department

Traffic Noises and Dangers

The nuisance of traffic noises and their possibly injurious effect have been described in previous letters The report to the minister of transport of a conference on traffic noises and priority of traffic at crossroads has been issued It recommends that a regulation should be made to deal with excessive and avoidable noise from automobiles that are badly constructed badly loaded or in faulty condition Some preliminary inquiries were made as to the types of horns in use and the practicability of framing a definition that would distinguish those which have a low and pleasing note from those which have a strident and irritating one It was found that the classification of horns into wind-blown (either hand or fan), electric-motor driven, and electric buzzer did not afford a basis for a regulation as it is possible to make horns of any of these groups with either objectionable or melodious notes The conclusion was that the only solution would be to permit specific types, e. g, bulb horns or other types that have been proved satisfactory by some test The conference held that the complaints against excessive and nerve racking noises from motor horns constitute a legitimate grievance and that the causes fall under two heads (1) unduly strident notes and (2) unnecessary and excessive using, particularly at night, during sleeping hours As to the first, a more powerful horn may be reasonable on the open road in rural areas where high speeds may be justifiable, certainly in the daytime, though at night the warning of the headlights should reduce the necessity to a minimum The second point is more difficult The conference explored the possibility of instituting zones of silence, where motor horns should not be permitted to sound at all or, alternatively, should be sounded only when there is some vehicle, pedesterian or animal in sight which necessitates use for the sake of safety This suggestion was uncompromisingly opposed by the motor organizations on the ground that nothing should be done to interfere with the discretion of the driver In the case of an accident, the first question asked by the police is "did the driver sound his horn?' An answer in the negative is usually regarded as indicating responsibility for the accident The conference recommended that the matter should be further investigated in consultation with the National Physical Laboratory, other scientific bodies and motoring organizations and manufacturers so as to fix a practical standard Another suggestion was that streets should bear adequate name plates, so that congestion due to drivers uncertain of their whereabouts slowing down and stopping in an effort to find out the name of the streets should be avoided

The problem of reducing the collisions at crossroads proved difficult. The law does not lay down any rule for motorists approaching crossroads and other junctions aside from the general prohibition against dangerous driving According to custom, motorists on an important road have the prior right of way and rely on the traffic from minor roads giving way to them The Automobile Association has for some years advocated the following courtesy rule, known as the offside rule whereby motorists are enjoined to look out for and give way to traffic approaching from the right But the conference did not approve of this rule. It thought that the best course would be that at road junctions traffic proceeding along a road of lesser importance should look out for and give way to that on roads of greater importance. The corollary is that signs should be placed at suitable points on the lesser roads warning motorists that they are approaching a road junction. The signs might take the form of an appropriate symbol with the words "Dead Slow" picked out with reflectors. In addition, there should be a white line across the road. The layout at road junctions was also considered. It was held that this should be different at a crossing of two roads of equal importance from that of the crossing of a major by a minor road. A complete roundabout was recommended for the first and a partial one for the second.

The Centenary of Thomas Young

This year is the centenary of the death of Thomas Young, one of the greatest physicists, who was also a physician. Of all the distinguished men whom the Quakers have contributed to science and to medicine, out of all proportion to the numbers of that sect, he was the most eminent. From his earliest years he showed extraordinary ability. At the age of 2 years he could read with fluency. He was only 20 when he wrote his celebrated treatise on the Mechanism of the Eye, which was read before the Royal Society, of which he was made a fellow in the following year. This paper dealt with accommodation of the eye. Later he gave the first description of astigmatism and formulated the three-color theory of color vision. He actually proved the existence of lenticular astigmatism in his own person. In physics his work on the interference of light renders him the main founder of the wave theory which ousted the corpuscular theory for a century. He introduced the term "energy" into physics. He made important contributions to the theory of the tides. He was professor of natural philosophy at the Royal Institution, a position which he resigned in 1803 to devote himself to medical practice, in which he was not a conspicuous success. However, he wrote a book entitled "Practical Nosology and Pharmacology," which was published in 1813. It is learned and still worth reading. His extraordinary versatility was further shown by linguistic talents, which he turned to account in translating the demotic text of the Rosetta stone before the Frenchman Champollion accomplished his great work on the subject. The inscription on the tablet to his memory in Westminster Abbey describes him as a "man alike eminent in almost every department of human learning."

High Death Rate from Cold Spell

The return of the registrar-general for England and Wales for the first quarter of this year shows that the deaths numbered 204,293, which is 88,654 more than in the preceding quarter and 67,978 more than in the corresponding quarter of 1928. The death rate was 21.0 per thousand of the estimated population, 7.1 above that recorded during the first quarter of last year. With the exception of the March quarter of 1919, when, owing to the influenza epidemic, the rate was 21.3, it was the highest rate recorded in any first quarter of the present century. It was due in a large measure to influenza and also to the high mortality from respiratory and circulatory diseases occurring during the spell of arctic weather in February. Influenza was either an immediate or contributory cause of death in 23,763 cases, or 11.63 per cent of the total deaths, whereas in the previous epidemic in the March quarter of 1927 the figures were 17,931 and 10.62, respectively. The mortality of infants under 1 year was 111 per thousand registered live births, 14 above the average of the ten preceding first quarters, and the highest in any quarter since the first quarter of 1919. The live births numbered 160,181, 4,512 more than in the preceding quarter but 7,745 below the number recorded in the corresponding quarter of 1928. They correspond to an annual rate of 16.5 per thousand.

DECREASE OF POPULATION

The natural decrease of population by excess of deaths over births was 44,112, against a natural increase of 43,386 in the corresponding quarter of 1926, a decrease of 1,786 in 1927 and an increase of 31,611 in 1928. This must not be taken to mean that this country has reached the stage of declining population, though, in consequence of the declining birth rate, the stationary stage seems in sight, but the birth rate and death rates have now so approximated that an unusually high death rate falls below the birth rate.

PARIS

(From Our Regular Correspondent)

May 29, 1929

New Researches on Alveolodental Pyorrhea

Dr. René Vincent has recently given a rather new explanation of alveolodental pyorrhea, and the therapeutic results to which it has given rise seem to justify a certain measure of recognition. He states that, in the gums, particularly in the region of the interdental "bridges," there is a kind of lymphoid tissue similar to that of the tonsils and the appendix. This tissue, he explains, serves as a sort of excretory organ of micro-organisms. The micro-organisms thus eliminated come from distant foci of infection, often located in the intestine. Having been carried to the region of the gums, they set up an inflammation, and this causes the localized necrosis of the maxillary bone and the fetid suppurations that are characteristic of pyorrhea. As a practical deduction of this mode of thinking, Vincent recommends ascertaining, in the blood taken from the region of the gums, what micro-organism is responsible for the disturbance and the preparation therefrom of an autovaccine. The use of such vaccine effects an arrest of the evolution of the pyorrhea and checks the primary causal infection.

Treatment of Erythremia

Erythremia, or Vaquez's disease, marked by an undue increase in the number of erythrocytes in the blood, has always been regarded as a grave disease combated with difficulty. MM. Vaquez and Mouquin have communicated to the Academy of Medicine three clearly characterized cases with remarkable results following use of phenylhydrazine hydrochloride. This substance, administered by mouth, with an initial dose of 0.001 Gm., which is gradually increased to 0.0015 and 0.0025 Gm., effects a rapid diminution of the number of red corpuscles, which, in a few days, drop down to the normal figure, while the number of leukocytes increases rapidly. This result having been secured (on an average, within two weeks), the treatment is followed only intermittently, afterward. In one of the cases cited, a cure has been maintained for a year. No untoward effects or incidents have been observed, other than a slight subicteric state. Nevertheless, M. Vaquez advises caution in the use of the drug.

An Eminent Preacher's Public Address on the Subject of Tuberculosis

The Reverend Father Sanson is at present one of the most eminent preachers in France. A priest of the Order of the Oratory, he has delivered, from the famous pulpit of the cathedral of Notre Dame de Paris, the special Lenten sermons for a number of years and with such success that crowds of people gathered at the church several hours before the portals were opened, and struggled to gain entrance. His eloquence is of a high order, and the clearness of his delivery is very impressive. All his sermons are transmitted by radio. He recently lent his support to a work of a social nature, the creation of a sanatorium for the tuberculous, to be located at Megève in Savoy. The meeting was held in an immense velodrome, used commonly for bicycle races and capable of seating more than 20,000 persons. High prices were charged for the seats, but they were all filled. He delivered a magnificent address, which was transmitted by loud speakers to the most distant parts of the vast arena The audience was astounded by the way he had been able to assimilate the medical conception and language needed to describe the march of tuberculosis and the evolution of the tubercle bacillus He closed his address with a pressing appeal for the vaccination of the new-born with the B C G vaccine, and it was generally admitted that his forceful utterances would have done credit to a member of the Academy of Medicine. A considerable sum was realized for the purpose cited

The Hygienic Aspects of Swimming Pools

M Dujarric de la Rivere has called attention in vigorous language to the insanitary conditions he has found in the municipal swimming pools of Paris as shown by the number of cases of conjunctivitis that develop among the bathers He has proposed to the Academy of Medicine a series of measures calculated to assure the constant cleanliness of the water in the pools The bathers should not be admitted to the pools until they have washed their feet in a small pool containing warm water in constant circulation A permanent filter should be installed that would purify the water of the pools two or three times a day The water on coming from the filters should be disinfected by means of sodium hypochlorite

A Court of Justice and Alleged Divine Intervention

A peculiar case was brought recently before the Tribunal de la Seine A woman who was preparing to take an examination to secure a position as a dental assistant accepted for more than a year, private instruction from a teacher She passed her tests successfully, but when her teacher demanded, as compensation for the instruction given, the sum of 2,400 francs ($96), as had been agreed on between them she refused to pay it. According to her statement, she had never been able to understand anything her teacher had tried to impart, and the evening before her examination, being certain that she would fail, she had made a pilgrimage to Lisieux—to the sanctuary of Saint Theresa—and had fervently prayed that she might help her Consequently, she was convinced that it was to the saint and not to her teacher that she owed her success The court seized with doubt and desiring possibly to accept, in part the theory of a salutary autosuggestion although declaring that the question of divine intervention had not been provided for in the laws, decided that the woman must pay her teacher but reduced the amount of the charge one half

Blood Transfusion Directly into the Heart

Dr Tzanck, physician to the hospitals of Paris made a desperate effort to save a patient in a state of acute anemia due to severe hemorrhages following abortion. Venous injection was impossible, owing to collapse of the veins and the patient was dying At this juncture, Dr Tzanck did not hesitate to make, with a long slender cannula a puncture in the fifth intercostal space, even with the sternum, and to inject 650 Gm. of blood directly into the heart. With the first 300 cc, the heart began to beat normally, and the patient made a most startling recovery

In Aid of Convalescent Heart Patients

A society has recently been founded in Paris with the avowed object of aiding poor patients suffering from heart lesions who have been dismissed from the hospital It will be known as the Fondation Dike in memory of the benefactress who first proposed the creation of such a society Dr Vaquez the French cardiologist, is the moving spirit, and the seat of the association will be the offices of the Hopital de la Pitie. The purpose of the society with respect to young subjects affected with organic disease of the heart, is to aid them in finding work in keeping with their physical capacities to watch over them during their apprenticeship, and to guide them in securing permanent positions With regard to older subjects the society plans to aid their families during the period of hospitalization and to find for them employment that is not too fatiguing These patients will be provided with a health record book, so that physicians who treat them, later on, may be acquainted with their personal histories and with the treatment that they have received As the result of the care that will be given them, they will have the moral satisfaction of knowing that their families are not suffering from want, and they will be encouraged by the thought that they will not, by reason of their lesions, be condemned to a precarious and miserable existence The minister of the interior, M Tardieu, who presided at the meeting for the purpose of organizing stated in his address and with good reason that the general public is under obligations to certain cardiac patients such as automobile drivers, truck drivers, railway engineers and aviation pilots, who have been prohibited from continuing the practice of their trade or calling, in order to protect the public from grave accidents that they might cause if a syncope should suddenly overtake them while on duty, as has occurred in numerous instances

Death of Dr Louis Guinon

Dr Louis Guinon has died at Paris, at the age of 69 Having specialized in pediatrics, he became the chief physician of two childrens hospitals (Trousseau and Bretonneau) He is the author of numerous articles published in the *Archives de medecine des enfants* and *Le Nourrisson* He was the founder of the Societe de pediatrie and of the Societe des amis des hopitaux des enfants He created many dispensaries for infants and at the close of the war, was entrusted by the Comite americain de l'Argonne with the organization and directorship of the Centre de Mandres

MADRID

(From Our Regular Correspondent)

May 16, 1929

Mortality from Tuberculosis in Spain

Dr Palanca, health inspector of the province of Madrid recently delivered an address before the Real Academia Nacional de Medicina on "Mortality from Tuberculosis in Spain" He mentioned the present enthusiasm for the creation of sanatoriums in Spain There are eight in the province of Madrid alone. A small town in the mountains has been wholly transformed into a tuberculosis colony and at present at Hoyo de Manzanares and in El Escorial two more sanatoriums are under construction. The speaker commented on the statistics of morbidity and mortality from tuberculosis which he thinks have been exaggerated Dr Codina admitted 50 000 deaths from tuberculosis yearly in Spain and calculated a morbidity of 500 000 The official statistics show a mortality from tuberculosis of 26 517 during 1928 as it was 29 991 during 1920, there is a decrease in the mortality The provinces which show the higher mortality from tuberculosis are Sevilla, La Coruña, Huelva, Madrid and Pentevedra while those which show the lower mortality are Teruel Segovia, Cuenca and Guadalajara The maximal mortality of 197 deaths from tuberculosis per thousand patients occurred in Sevilla and the minimal mortality of 57 deaths per thousand in Teruel The speaker excluded the climate as a factor in the mortality from tuberculosis He considered as important factors the increase in population and industries He did not regard the high prices of food as a big problem, and asserted that the market in Madrid is one of the most economical in Spain. He concluded that in the prophylaxis of tuberculosis all the efforts of the state provinces and cities should be united. The work should be provided for by legislation the selling of milk should be regulated a campaign for improved hygiene should be made and a school of phthisiology should be opened. There is a greater need for healthful and inexpensive lodgings with plenty of sun and light, where tuberculosis may be prevented, than for the construction of sanatoriums

Delivery of Five Fetuses

Prof Isidoro de la Villa recently lectured on a case of multiple delivery, which he observed in his clinic at the Faculty of Medicine of Valladolid He mentioned that anthropoid female monkeys have two or three offspring at one time but that in the human being the function has been established in such a way that the woman has only a monthly ovulation and becomes pregnant regularly with only one child In the case reported, a woman, aged 32, had had four normal children at different deliveries, then two abortions of twins, and finally the reported pregnancy Labor started at the sixth month of pregnancy The first fetus born was a male, in vertex presentation In the evening of the same day the patient's temperature rose to 38 5 C (99 3 F) and she was admitted to the hospital of Valladolid She developed a serious infection and on the next day her temperature reached 40 C (104 F) Dr Muñiz, an assistant professor on the faculty of medicine, punctured the second bag of waters, and a second male fetus was born in breech presentation Then with no intervention, and at intervals of approximately half an hour, two other female fetuses were born, one of them in vertex presentation and the other in breech presentation All the fetuses were born alive. Three hours later, the placenta and membranes were delivered Two and a half hours later, the last female fetus was born, enclosed in its membranes A mass consisting of three placentas was then delivered The fetuses were all of similar size, measured from 30 to 35 cm in length, and weighed about 800 Gm They were well developed and lived from one to six hours Three of the fetuses had their own membranes and independent placentas (multiovular), and two of them, probably the two males, had a common placenta and chorion, and were separated only by means of individual amnions (uniovular) The mother had a serious puerperal infection, but she recovered The speaker recognized, as causes of multiple gestations heredity, multiparity, age and climate Multiple gestations are frequently observed in families with mental diseases and in women with anomalies and malformations of an atavic nature They may be due to the father s influence Sexual maturity is the most opportune time for these pregnancies, which generally are observed in persons more than 30 years old The influence of climate is noticed in Russia Sweden France and Spain The occurrence of twin births in Castile is not rare Professor Villa knows of a delivery of three children and another of four, which occurred in the last few years Five or six fetuses in a delivery are seldom viable Prolific deliveries represent racial backwardness and a return to a type of life for which the women of today are not prepared It is preferable that only one fetus be born and that it be healthy and strong

Arthritis Deformans

Dr Bastos delivered a lecture at Dr Marañon s clinic on arthritis deformans His work was devoted to those forms of chronic rheumatism which present pronounced local lesions and hence become surgical It is necessary to know these rheumatic manifestations because they are of considerable importance For each patient with tuberculosis there are three with rheumatism and this ratio which is large enough in itself is still higher in some regions of Spain On the other hand it happens that processes which are far from being rheumatic processes are diagnosed as such Concerning arthritis deformans the author said that the microscopic lesions are appreciated in the first stages of the disease These lesions produce regression of the cartilage, which is transformed into fibrous cartilage and then the development of hemorrhagic synovitis Concerning the pathogenesis of arthritis deformans the speaker cited the mechanical theory which attributes the lesions of this disease to the wear of the articular surfaces, and the infectious theory, which regards the disease as a form of rheumatism produced by focal infection Lastly, the speaker considered the constitutional factors, which contribute to the premature aging of the articulations, and the influence of certain endocrine conditions in the production of arthritis deformans

Prize for a Paper on Medical Deontology

Dr Sanchiz Banus, president of the College of Physicians, was recently honored by the physicians of Madrid at a banquet A subscription fund was begun for a prize to bear his name, which will be devoted to the best paper written during the year on the following phase of medical deontology operation of medical colleges, or social and corporative medicine

Amplification of the Cancer Institute

The Cancer Institute, with Dr Jose Goyanes as its director, has been enlarged by two new buildings One of them is devoted to laboratories while the other has been provided with seventy beds for cancer patients from the province of Madrid. The building has been financed by the provincial deputation of Madrid The ceremony of the inauguration was presided over by the queen, who is president of the league against cancer

Central Dispensary of the Red Cross

The Central Dispensary of the Red Cross, under the patronage of the king and queen, has been opened for service The Royal family and prominent government officials attended the opening ceremonies The new building, which cost 5,000,000 pesetas (about $1,000 000), is one of the best that the Red Cross possesses It is a masterpiece of architecture Provisions have been made for the installation of several clinics

Closing of Several Universities by the Government

General Primo de Rivera, as the head of the government, has aided the general progress of Spain Public instruction and hygiene have been improved, and large amounts of money have been provided for schools, sanatoriums, parks, dispensaries, hospitals, roads and ports His policy, however, has caused dissatisfaction and some disturbances among the students The students of the University of Madrid protested vigorously against the permission given by the government to men of religious sects to award scientific titles and to act as jurors at the examinations of the students The government, without giving any attention to the reason for the protests, demanded the obedience of the students, but they disobeyed the order The police were ordered to the universities, but this act was considered, both by students and by teachers, as a violation of the rights of the university The police fired into the air, wounding a man, but not seriously The government then ordered the university to be closed until the fall of 1930 The students who wish to take their examinations should pay their tuition at some other university and be examined there The students at the Universities of Barcelona (the second in Spain) and Oviedo made the same protests and these universities also were closed by the government In Santiago de Galicia and in Valencia, the faculties protested, and the same punishment was imposed on these universities In Valladolid where the disturbances have been of minor importance the teachers have imposed another payment of tuition on the students The students of medicine and the women students were not guilty of these disturbances, but they were also obliged to pay tuition again in order to be examined Some of the professors who would not stand this state of affairs, resigned others have protested in writing and still others have declared adherence to the policies of the government The parents of the students were affected by these disturbances more than any one else as they were obliged to make a repayment of the tuitions They hold the teachers responsible for exerting influence on the students In connection with the official inauguration of the universal expositions of Sevilla and Barcelona the government has resolved to pardon the students Thus the universities have been reopened, and university life is going on as usual

BUCHAREST

(From Our Regular Correspondent)

May 27, 1929

The Sight of Motor Car Drivers

According to Prof Dr Emile Brosz, managing director of the postgraduate medical courses in Hungary, opinions differ as to the minimum of eyesight which should be demanded of motor car drivers Some authorities believe that motor car drivers should possess visual acuity equal to that of locomotive engineers, others believe that less is sufficient It has been suggested that drivers be permitted to make written statements as to their fitness for driving, after which they would be held responsible for accidents and the like on the basis of these statements The chief of police of Paris has stated that he cannot demand proof of the physical soundness of all applicants, as the medical examination of more than a hundred applicants for a driver's license daily would entail too much time and expense Competent men, however, are urging that conditions of fitness be laid down and that a thorough medical examination of the candidates be made in every case. The question is deemed so important that the Belgian and French medical academies are dealing with the matter seriously and have advocated detailed instructions They are not in force, however Professor Grosz says During the war, military commandants enforced very strict rules as to who was competent to drive military motor vehicles, this was justified, as these cars carried many soldiers Since the termination of the war the French academy has repeatedly dealt with this problem, and one of its reports characterized the situation as follows 'To become eligible for political voting one must be 21 years of age, but any urchin suffering from bodily defect may run down pedestrians ' This assertion made a deep impression, as just at that time two academicians were fatally injured in automobile accidents and yet neither the efforts of the academy nor those of the medical society were successful Finally, the medical members of the chamber and the senate were mobilized but they also failed in their efforts It is noticeable also that England and Germany have failed to lay down conditions of fitness Switzerland demands at least 5/10 visual acuity but does not exclude drivers with only one eye The same rule holds true in Italy In Norway a distinction is made as to the visual acuity acquired better vision is demanded of professional drivers than of other drivers Professor Jeandelize compiled the conditions demanded in the different countries and made the proposal to fix the minimum of visual acuity in the better eye at 5/15 with 5/100 acceptable in the other eye

In Hungary the issue of a license is based on a medical certificate given by a city medical officer, and if he does not find the sight to be satisfactory he has to appeal to the state ophthalmologic hospital for a decision The measurement of visual acuity, however, is not regulated The police may refuse any applicant The applicant then has the right to appeal to the ministry of internal affairs in which case the ministry asks for the opinion of the supreme health board through the ministry of public welfare.

The health board maintains that if the vision of one eye is perfect or at least 5/7, a man with only one eye may get a driver's license It is necessary that the conditions of fitness for drivers should be the same as for individuals in the railway and military services Professor Grosz believes on the ground of experiences gained abroad and with due respect to inland conditions, that it would be appropriate to fix the minimal acuity of the better eye at 5/10 if the vision of the other eye is as much as 5/20 If the sight of the other eye is less than 5/20, the vision of the better eye should be at least 5/7, with normal visual fields and perfect color vision In his opinion when one eye is missing the matter should be settled individually The loss of one eye is prohibitive in the case of a driver of an autobus and in others who carry passengers professionally, while it is permissible in the case of a driver who owns his car He urges periodic examinations of all drivers Motor car drivers are exposed to different injuries, which eventually may seriously interfere with their sight and other bodily and even mental functions, therefore semiannual or annual examinations are indicated In order to settle the question in Hungary, Professor Grosz is going to ask the Society of Hungarian Ophthalmologists to discuss the matter and to pass on resolutions that are to be submitted to the national assembly

The Perception of Changes in the Weather

At the recent meeting of the Royal Medical Society, Dr Z Dalmady read a paper on the perception of changes in the weather He said that some men, particularly sick ones, can predict changes in the weather Case histories show harmony between the weather and the condition of some patients, and statistics show an increase in certain diseases in connection with the weather The feeling of an oncoming change in the weather is a presentiment of local atmospheric conditions, which must be distinguished from sensibility to a change in the weather Weather feeling men may not be able to predict the exact change in the weather The factors that act on these men must be sought among those atmospheric qualities which are experienced in a closed room as well as by clothed men in the open According to recent observations, the presentiment of a change in the weather depends on the exchange of air masses The determining factors may be sought in the constitution of an air current, in the nature of its suspensions and in its electrical qualities These factors may have important roles in changing the physical features of the air It is probable that the presentiment of a change in the weather pertains to allergic phenomena, and in its establishment a decisive role is played by the physical features of the air

Dr M Farkas remarked that every one is more or less of a "weather feeler" On bright, sunny days the sick man feels better, and the healthy man is refreshed—his temper is calm and his ability to work is greater The powers of observation of blind children are keener on bright days than in cloudy or rainy weather Rheumatic gouty and tabetic patients display a special sensibility toward a change in the weather, but presentiment means something different namely that some patients, and even healthy individuals, owing to certain bodily sensations, are reliably able to predict a change in the weather, from thirty six to forty-eight hours in advance The sensations cease when the rain, snow or wind storm begins and in the interval they are quite devoid of symptoms According to his observations there is no connection between the barometric reading and the presentation of weather feeling During the war Farkas observed the weather feeling of soldiers with amputations, shot wounds and bayonet wounds and found that specific sensibility for a change in the weather was displayed by patients in whom the periosteum was adherent to its base and was immovable The presentiment of weather change a feeling undoubtedly of neuropsychic importance is attributed by Stein to the expectation feeling, which is regarded by the Norwegian philospher Aaro as a new elementary spiritual phenomenon According to him certain animals and men possess the ability to foretell changes in the weather as a result of reminiscences, mind pictures and the like laid down in the conduction paths and condensed into instincts This theory makes it purely a psychic phenomenon which is not acceptable. It is more rational to assume that the presentiment of a change in the weather is the feeling of physiologic and pathologic processes going on in the body as the result of accumulated influences of changes in the weather The confirmation of the connection is a logical process Farkas believes that the problem of weather feeling will come to play a prominent role in the numerous problems of psychology and philosophy under dispute, because physical and psychic manifestations are presented in this particular feature, and their explanation eventually will be common and simple

BERLIN

(From Our Regular Correspondent)

June 1, 1929

Meeting of the Deutsche Gesellschaft für Chirurgie

(Continued from page 2183)

In the general discussion, Tonnies of Würzburg emphasized the good results secured with puncture (sometimes with lavage) in infants and young children He reported a series of sixty-one cases with three deaths (5 per cent) In some of the patients a later thoracotomy was required, which was then well borne Schönbauer of Vienna displayed an apparatus recommended by Demel for the aspiration of the purulent effusion It consists of two curved trocars, which, joined together to form a circle, are connected with a tube that leads to a bottle The suction device has given good satisfaction Heller of Leipzig performs rib resection in severe empyemas, under differential pressure then closes the wound and drains by the Iselin method through a newly created puncture wound into a wine bottle In eighty-one cases he had a mortality of 24 per cent The average period of treatment was from six weeks to two months Only in a few cases did small plastic operations become necessary on account of residual cavities Hosemann of Freiburg recommended for the operation on a tuberculous total empyema associated with a mixed infection the replacing of the prevertebral rib resection by a parasternal resection which gives better results without the operation being thereby materially enlarged in scope He has performed the operation seven times without a fatality One patient, however later succumbed to amyloid degeneration Hauks of Breslau gave an account of his experiences with the use of paraffin fillings in tuberculosis of the upper lobe He has employed the method thirty two times during the past two and a half years thirteen times in extensive bilateral processes and nineteen times in unilateral tuberculosis of the upper lobe in which latter cases partial compression is sufficient Pneumolysis must be carefully carried out before the paraffin filling is applied One can then feel the cavities, which are loose and flabby to the touch The use of the paraffin fillings depends on the success of pneumolysis The method could not be applied in ten patients all of whom succumbed to the disease Of the successful cases one ended fatally on the third day from meningitis An infection of the filling bed developed in one instance Of the thirteen bilateral cases, five remained unchanged four will doubtless prove fatal and three show improvement, while one patient is free from expectorations and from bacilli The nineteen unilateral cases present a more favorable result Nine patients are free from expectorations and from bacilli six are improved, three are unchanged one is worse, and one died soon from meningitis (as already mentioned)

Sauerbruch emphasized that the fundamental idea of extrapleural plastic operations is the contraction of the whole lung and therefore the filling with paraffin should contract the whole lung But in bilateral pulmonary tuberculosis to which some of the reported cases belonged a partial contraction must of course suffice There are however grave dangers associated with the paraffin filling method Perforation may result after five seven and even eleven years A grave danger is associated with the method in exudative tuberculosis since, following the implantation of the paraffin fillings a more rapid general intoxication may intervene The local reaction on the right auricle and the right side of the heart may be so great that it will become necessary to remove the paraffin filling It is also very difficult to decide whether the compression is sufficient However, the paraffin filling is the best type of filling as yet discovered.

GEOGRAPHIC AND SOCIAL INFLUENCES IN VARIOUS DISEASES

Professor von Haberer of Düsseldorf discussed geographic and social influences in relation to the various diseases with which he had become familiar during his seventeen years of activity in the Alpine regions of Austria (Tyrol, Styria, Salzburg, Carinthia) and in his present field of activity (Düsseldorf) In Tyrol there were few cases of cancer, whereas in Styria many cancerous conditions were observed, especially in the capital city (Graz) Whereas in the Alpine regions there was a considerable number of goiters, there were, in contrast with Dusseldorf, few cases of exophthalmic goiter With reference to other diseases, many peculiar differences were noted Whereas in the Alpine regions von Haberer could make frequent use of local anesthesia, in Düsseldorf, on account of the marked sensitiveness of the population (men as well as women) he had to give it up almost entirely and resort more to inhalation anesthesia He emphasized also the entirely different mentality of the patients in the industrial region about Dusseldorf, which expressed itself in a different attitude toward their ailments and their physician, for instance, in the lack of "will to recover' that is evidenced by the population entitled to the benefit of social insurance This attitude was observed more particularly in his patients operated on for gastric or duodenal ulcer In Innsbruck or Graz, about 95 per cent of such patients recovered and were free from all symptoms, while in Düsseldorf he could note a difference between the so-called private patients (who were well satisfied with the operative result) and the patients who were insured in the *krankenkassen* The majority of the latter class complained, after the lapse of varying periods following the intervention, of discomforts for which a thorough examination revealed neither clinical nor roentgenologic basis Haberer called attention also to the peculiar gravity of the disorders of the biliary tracts, as encountered in the industrial region which induced him to give up almost entirely closure without drainage, which he had been able to employ in the Alpine regions in 70 per cent of the cases, by reason of the gravity and frequency of the accidents and the great number of postoperative pulmonary disorders and embolisms

URETERAL CALCULUS

Professor Voelcker of Halle presented a comprehensive paper on ureteral calculus He distinguished two large groups of cases the aseptic and the infected cases The former often develop unexpectedly, an attack of colic being the first symptom, and may be difficult to distinguish from cholecystitis, appendicitis ileus and the like especially if they are located on the right side A roentgen diagnosis is not always possible and the characteristic symptom of microscopic hematuria occurs also in appendicitis But for the operative indications the diagnosis is of the greatest importance since in renal calculus an immediate operative intervention as in the other diseases mentioned is not an open question The question as to the treatment of acute aseptic renal calculus may be solved by spontaneous elimination of the stone or the colic may stop although the stone has not been passed. The checking of the colic is often occasioned by the blocking of the ureter for according to Voelker, the colic is caused chiefly by the stasis of urine. The stoppage is often caused by the lodging of a stone at one of the three natural narrow places of the ureter The result is the swelling of the kidney In such cases a cystoscopic or chromocystoscopic examination should be made, and above all ureteral catheterization should be employed. Often that alone will effect a passing of the stone or cause the stone to be pushed into the renal pelvis and thus eliminate the stoppage of urine and the colic If all measures fail (possibly including the injection of spasmogenic remedies into the ureter and paravertebral anesthesia), ureterotomy, with subsequent primary suture, is the preferred operation in these acute aseptic cases If the stoppage of urine and colic cease without the stone being passed the picture of chronic aseptic ureteral calculus is produced While it is true that such stones often may be carried for years without any particular harm to the kidney resulting, and that they may be spontaneously passed after many years, it is nevertheless to the advantage of the carrier if its removal can be effected for the pathologic aspects of the condition may come to resemble those of gallstones Colic-free periods may alternate with severe attacks of colic and there is constant danger of infection and damage to the kidney These attacks are sometimes referred to as attacks of renal calculus, which is not quite accurate, since the stones do not cause colic, as a rule, until they descend into the ureter Voelcker discussed the various diagnostic aids rectal and vaginal examination, cystoscopy, ureteral catheterization, wax sounds that take an impression of a stone if present and acoustic sounds (both uncertain procedures), and roentgen examination By way of treatment one may consider lavage of the kidneys with large amounts of fluids (mineral water, from one to two liters of tea, with addition of from one to two tablespoonfuls of glycerin, the urine to be held as long as possible and then quickly evacuated), use of antispasmodic substances (atropine, morphine, and hypophysin, which stimulates peristalsis but apparently does not act directly on the ureter) and the subaqueous intestinal bath He mentioned also the injection of oil or glycerin into the ureter, various analgesic remedies, including papaverine, and procedures of instrumentation, principally the gradual dilation of the ureter by the Burger method He was inclined to disparage other instrumental measures Voelcker rejects also the use of the retention catheter in the ureter The treatment may be extended over a long period but care must be taken that no renal injury, infection or hydronephrosis develops There may be primary infection due to ureteral calculus, or secondary infection Frequently, phosphates are involved, which have a rough surface and hence do not pass out so readily They cause stoppage of the urine less frequently, and, because of the inflammatory changes in the ureteral wall, they are held less tightly As they are, for the most part calcareous the diagnosis is facilitated by the use of the roentgenogram Here, too, a distinction must be made between the cases with and without stoppage of the urine In the latter cases, internal treatment is usually ineffective, and endovesical treatment is not to be recommended Thus, after exact testing of the functioning of the organs involved operative treatment is to be considered If the kidney is to be preserved, ureterotomy is indicated Operative intervention is easier in the infected cases, by reason of the somewhat thickened ureteral walls, than in the aseptic cases If the kidney cannot be preserved, it must be removed In these cases, one need not bother about the stone at first—it can be removed later In the cases of acute stoppage of the urine, in which the patient s life is threatened, if it is not possible to bring relief with the ureteral catheter, attention must be centered on the kidney and if it cannot be preserved it must be removed or a renal fistula must be constructed and no attention need be paid for the time being to the stone, which can often be removed by suction, through the constructed fistula The most difficult cases are those with bilateral calculi, which may lead to complete anuria In aseptic cases complete anuria may be endured up to fourteen days If the roentgenogram and the urologic examination do not make it evident which side is more gravely involved and the anamnesis does not help to decide, both kidneys must be exposed and the one that after palpation, appears to be more seriously involved must be operated on Voelcker discussed then, the operative technic for the exposure of the ureter in its various portions, and recommended the median incision for deep lying stones

(To be continued)

Marriages

Walter Drury Hawkins, Harrisburg, Pa., to Miss Elizabeth Shorkley Hobart of New Cumberland, April 6

Ralph Shaw Graham, Elyria Ohio, to Miss Elizabeth Frances Herr, at Moorestown, N J, June 15

Richard Dale Hopkinson Jenkintown, Pa, to Mrs Leona Alexander Davis of Philadelphia, April 24

Lodovico Mancusi-Ungaro, Newark, N J, to Miss Frances Groves Chambers, at Philadelphia, June 12

Richard D Evans, Santa Barbara Calif, to Miss Elizabeth Bristol of Long Island, N Y, recently

F Willis Almond Boise, Idaho to Miss Florence Maye Hardisty of Jennings, Kan., March 17

Alec McMurchie Agnew to Miss Pauline Creelman, both of Vancouver, B C., Canada, May 3

George Swartz Klump to Miss Elizabeth Marsden Winner, both of Williamsport, Pa, May 15

William D Laswell, Kings Mountain, Ky, to Miss Lucile M Young of Highland, June 5

Mark Greer to Miss Oris Camille Klasing, both of Vandalia, Ill, at New York, June 14

David J Hetrick, Harrisburg, Pa, to Miss Gladys Kerlin of Altoona, May 1

Mead Burke Madison, Wis, to Dr Myra Emery of Kenosha, May 18

James D Brittingham, Philadelphia, to Miss Janette Wallace, June 12

Arthur B Light, Philadelphia, to Miss Mary Glover of Boston, recently

Judson I Doss to Miss Artie Bolin, both of Milton, Ill, June 7

Deaths

Edgar Orrin Crossman ⊕ Washington, D C., University of Vermont College of Medicine, Burlington 1887, medical director of the U S Veterans Bureau member of the American Psychiatric Association and the New England Society of Psychiatry, member and past president of the New Hampshire Medical Society, formerly professor of mental diseases at his alma mater, aged 64 died, June 21, at his summer home in Bedford, N H, of pneumonia

Albert Richard Thomas, New Orleans, Tulane University of Louisiana School of Medicine New Orleans, 1919, member of the Louisiana State Medical Society, formerly assistant in clinical gynecology at his alma mater, served during the World War, aged 36, resident surgeon at the City Hospital for Mental Diseases where he died suddenly, June 9 of heart disease, six days after an operation to remove a collar button which had lodged in his lung

Frederick Andrew Bonthius, Pasadena, Calif, Northwestern University Medical School, Chicago, 1902, at one time clinical assistant in medicine at his alma mater, served during the World War, formerly on the staff of the Los Angeles General Hospital, aged 55, died, April 6, in a local hospital, of metastatic carcinoma of the liver

Thomas Bernard Carpenter ⊕ Buffalo, Harvard University Medical School Boston 1890 associate professor of genito-urinary surgery, University of Buffalo School of Medicine formerly city bacteriologist on the staffs of the Buffalo General Hospital and the Buffalo City Hospital, aged 60, died, May 27, of tuberculosis

Albert Earl Mowry ⊕ Chicago Northwestern University Medical School Chicago, 1898 formerly on the faculty of his alma mater member of the American Urological Association veteran of the Spanish-American and World wars on the staff of the Provident Hospital, aged 55 died, June 16, from hemiplegia

Charles William Bartlett, Tampa Fla University of Maryland School of Medicine, Baltimore 1893 member of the Florida Medical Association past president of the Hillsboro County Medical Society veteran of the Spanish American War city health officer aged 59 died suddenly May 29, of heart disease

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⊕ Indicates Fellow of the American Medical Association functional, or from subjects undergoing laryngectomy for carcinoma of the larynx. Thyroid glands from freshly killed sheep were pooled.

Details concerning the patients are given in Table 1. The case numbers identify individual patients, in some of whom more than one type of tissue was studied. The tissue diagnosis is based, in each instance, on histological examination. In case 2, the *normal thyroid tissue was infiltrated with anaplastic thyroid carcinoma.* Analysis of a lymph node metastasis from this tumor indicated that it had minimal function in comparison to the thyroid gland. In case 5, the normal thyroid was the site of metastasis from an adenocarcinoma thought to be of prostatic origin.

Case 7 was clinically euthyroid, although the thyroid gland showed moderate hyperplasia. The tissues listed as "nodular goiter" comprise a heterogeneous group in which a more specific diagnosis could not be made. The thyroid architecture, although for the most part well differentiated, exhibited much pleomorphism in terms of cell height, amount of colloid, shape of the follicles, etc. In cases 9, 10 and 11, portions of the goiter which *appeared different macroscopically were analyzed separately. None of these* glands had been associated with clinical hyperthyroidism or hypothyroidism.

The tissues listed as "follicular adenoma" were characterized by their occurrence as discrete, encapsulated areas of abnormal tissue in an otherwise normal gland. The fetal adenoma was of microfollicular type and involved almost the entire thyroid gland.

The tissues listed as "carcinoma" were obtained from metastases in the neck which were clearly outside of the thyroid gland except in case 16. In the latter the large, anaplastic tumor contained scattered follicles which may have been normal thyroid tissue. In case 15, the normal thyroid had been ablated when the patient had received 250 mc. of I^{131} for therapy of other metastases 6 years previously. Surgical excision of recurrent tumor was demanded by tracheal obstruction. In case 13, the residual thyroid gland remaining from partial thyroidectomy 23 years previously was removed and found to be abnormal but, apparently, not carcinomatous. It is listed as "nodular goiter." The follicular carcinomas in both case 13 and 15, although predominantly well differentiated, displayed the usual pleomorphism and contained areas of anaplastic epithelium.

Labeling of these tissues was performed *in vivo* by the oral administration of radioiodide, or *in vitro,* as indicated in Table 1. In cases 2, 7 and 12, some of the *in vivo* labeled tissue was also incubated *in vitro* with I^{131}. Since the latter procedure introduced a much higher quantity of radioiodine, it was possible to study both *in vivo* and *in vitro* labeled iodoproteins in the same tissue.

In the *in vitro* procedure, fresh tissue slices approximately 0.2 to 0.5 mm. thick were incubated in an oxygenated Krebs-Ringer phosphate medium, pH 7.4 at 37° C in an oscillating water bath. The duration of incubation is given in Table 1. The tissue slices: medium ratio varied from 150 to 330 mg./ml. Except in cases 3, 7 and 11, however, the quantity of I^{131} accumulated by the slices, as given in Table 1, was measured in separate experiments in which the ratio was 35 to 70 mg./ml.

The *in vivo* radioiodine doses were from 50 to 300 μc.; the time between dose and tissue removal are given in Table 1. The quantity of I^{131} accumulated *in vivo,* as listed in Table 1, was determined by a separate tracer dose given a short time before the experimental dose, and accumulation was measured at 24 hours.

The methods employed for fractionation and analysis of iodine-containing component of the tissues were the same as those described before (1).

RESULTS AND DISCUSSION

The quantity of radioiodine accumulated by the human thyroid tissues, and the proportion of this iodine which was in organic form, are listed in Table 1. The latter figure represents radioiodine which did not move from

TABLE 1. CLINICAL DATA AND RADIOIODINE ACCUMULATION BY HUMAN THYROID TISSUE

Case No.	Thyroid tissue diagnosis*	Age (years)	Sex	Method of labeling	Time of labeling (hours)	Total tissue I^{131}†	Organic I^{131} (% of total tissue I^{131})	Particulate iodoprotein¶ (% of organic I^{131})	Front running component (% of particulate I^{131})	Electrophoretic mobility of thyroglobulin#
1	Normal	53	M	*In vivo*	48	27‡	100			
2	Normal	33	M	*In vivo*	48	28	100			α-1
				In vitro	2.5	19	24			α-1
3	Normal	54	F	*In vitro*	4	5.7	32			slow α-1
4	Normal	30	F	*In vitro*	4	—	83	1.2	4	α-1
5	Normal	71	M	*In vitro*	3	—	78	5.6	9	
6	Normal	60	F	*In vivo*	24	—	100	>4, <7	0	
7	Diffuse Hyperplastic Goiter	27	F	*In vivo*	72	50	100			slow α-1
				In vitro	4	3.2	9			slow α-1
8	Chronic Thyroiditis (Hashimoto's)	50	F	*In vitro*	8	12	25			α-1
9a§	Nodular Goiter*	34	M	*In vitro*	4.5	51	85			slow α-1
9b					4	26	44			slow α-1
10a	Nodular Goiter	45	F	*In vitro*	4	29	78			α-1
10b					4	22	88			α-1
11a	Nodular Goiter	54	F	*In vitro*	4	4.0	71			inter α-1-2
11b					4	—	54			slow α-1
12	Nodular Goiter	37	F	*In vivo*	24	29	100			
				In vitro	3.5	27	92			slow α-1
13 (1)	Nodular Goiter	35	M	*In vitro*	4	—	20	5	3	α-1
1	Follicular Adenoma			*In vivo*	48	‡	100			
3	Follicular Adenoma			*In vitro*	4	5.0	26			inter α-1-2
4	Follicular Adenoma			*In vitro*	4	—	77	4.8	5	slow α-1
14	Fetal Adenoma	19	M	*In vitro*	5	—	5			slow α-1
15	Follicular Carcinoma	66	F	*In vitro*	4	2.4	31			α-1
13 (2)	Follicular Carcinoma			*In vitro*	4	—	12	37	<3	slow α-1
2x‖	Anaplastic Carcinoma			*In vitro*	2.5	2.1	2			
2y					5	2.3	11			
16	Giant and spindle cell Carcinoma	82	F	*In vivo*	144	—	—	5.9		

* Cf. text for further details.

† *In vivo* = % of dose at 24 hours. *In vitro* = % of total I^{131} added/100 mg. tissue.

‡ Proportion of total uptake accumulated by the adenoma is not known.

§ Letters a and b refer to macroscopically different portions of the same tissue.

‖ Letters x and y refer to tissue removed at different times.

¶ The value for particulate iodoprotein includes the front running component (cf. text).

# Given as the serum globulin zone coincident with the radioiodine peak (cf. text).

the origin on chromatography, and was shown by hydrolysis to be composed of iodoproteins (cf. below). Iodide comprised almost all of the remaining radioiodine. In the *in vitro* experiments, it is not known what proportion of this iodide was due to mechanical occlusion only. Smaller quantities of other radioiodine components were sometimes found on the chromatograms, as described in the previous paper (1).

PARTICULATE IODOPROTEIN

In the early phases of this work, the existence of a thyroid radioiodine fraction insoluble in 0.15 *M* NaCl was unsuspected. Consequently, in only 7 of the cases studied was this "particulate" radioiodine measured. In one follicular carcinoma, the value of 37% for particulate iodoprotein was similar to that found in rat thyroid tumors (1). In the other tissues, normal or abnormal, the values were in the vicinity of 6% or less. Such small values might be considered insignificant and due to procedural artifacts. The results of hydrolysis (cf. below), however, revealed differences in iodinated amino acid constituents between the particulate and the soluble iodoproteins in about half of the cases.

Separation of the tissue homogenate into subcellular particulate fractions was performed in case 4, a normal thyroid gland, by means of the two-phase sucrose technique of Hogeboom, Schneider and Striebech (2), employing added $CaCl_2$. The "nuclear" fraction contained 3.8% of the organic iodine in this tissue, which had been labeled *in vivo*. This finding requires confirmation.

Chromatographic analysis of the particulate fraction was performed in all but one instance. Almost all of the radioiodine remained at the origin, 3% or less was iodide, and from 0 to 9% moved with the solvent front (Table 1).

SOLUBLE IODOPROTEINS—HUMAN THYROID TISSUE

Salting-out curves in phosphate buffer (equimolar KH_2PO_4 K_2HPO, pH 6.5) were carried out with the soluble iodoproteins from each of the tissues. In about $\frac{1}{3}$ of these, a 0.15 *M* NaCl extract of the tissue was used as starting material. In the others, the supernatant phase of tissue homogenized in 0.15 *M* NaCl was used. In two instances (case 4—normal, case 11), separate salting-out curves of extract and supernatant were not significantly different. All curves except for that in case 16 were performed with added normal human serum.

A typical salting-out curve is illustrated in Figure 1. Most of the soluble iodoprotein precipitated at low buffer concentrations in a manner typical for thyroglobulin. In Table 2, the buffer concentration at which each salting-out curve exhibited its steepest slope is listed. These values ranged from 1.54 to 1.68 *M* in the normal human thyroids (mean 1.60 ± .05),[2] and from 1.50 to 1.68 *M* in the abnormal tissues (mean 1.57 ±.05). The overall mean was 1.58 ±.05. In the carcinoma from case 2, a value is not given since the salting-out curve was very much different from all the others. Apparently this tissue did not incorporate I^{131} into thyroglobulin (cf. below). It seems likely that the variations between individual curves were due to experimental error, and that the solubility of thyroglobulin from abnormal tissues was not significantly different from normal. In those experiments in which normal and abnormal tissue were examined in the same subject (cases 1, 3 and 4), the slope maxima agreed closely. Comparison of thyroglobulin labeled *in vivo* and *in vitro* showed no significant difference in case 2-normal and case 12, but the latter appeared to be less soluble in case 7.

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[2] Mean ± standard deviation.

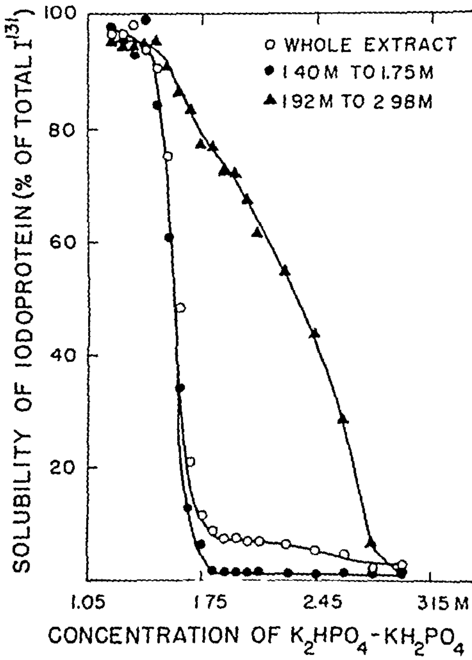

FIG. 1. Salting-out of soluble iodoproteins in the follicular adenoma from case 4. The solid symbols represent iodoprotein fractions isolated by precipitation between the indicated limits of KH_2PO_4-K_2HPO_4.

The salting-out curve of the tissue extract in Figure 1 also demonstrates the presence of a small amount of iodoprotein which is more soluble than thyroglobulin in phosphate buffer. Quantitation of this material is indicated in Table 2, where it is listed as "thyroid S-1 iodoprotein." The values were calculated by subtracting the iodoprotein soluble in 2.98 *M* phosphate buffer from that soluble in 1.89 *M*. In seven instances, salting-out curves were performed on undialyzed extracts or supernatants labeled *in vitro*, whereas all other curves were obtained either with *in vitro* labeled material which had been dialyzed against a large volume of .001 *M* phosphate buffer, or on *in vivo* labeled material. As indicated in Table 2, it was necessary to correct the S-1 iodoprotein values in these seven instances for the iodide

TABLE 2. SALTING-OUT OF SOLUBLE IODOPROTEINS IN HUMAN AND SHEEP THYROID TISSUE

Case No.	1.19 M	1.4 M	1.89 M	2.98 M	S—1	Slope maximum
			Normal human			
1	100	97.0	7.0			1.68
2 (*in vivo*)	99.0	91.0	9.9	5.0	4.9	1.58
2 (*in vitro*)	98.5	84.7	11.1	4.4	6.7	1.54
3	100	92.1	7.6	3.6	4.0	1.54
4	95.7	94.0	2.6	1.5	1.1	1.61
5	96.2	94.6	5.0	2.3	2.7	1.61
6	92.0	88.0	7.1	5.7	1.4	1.61
			Abnormal human			
7 (*in vivo*)	104	97.8	9.6	9.9	0	1.64
7 (*in vitro*)	93.8	82.2	6.6	5.8	0.8	1.50
8	105	88.0	8.8	1.9	6.9	1.50
9a	97.4	89.8	32.1	26.6*	(2.5)	1.58
9b	97.2	91.4	56.2	46.3*	(4.8)	1.50
10a	100	102	46.5	40.0*	(2.1)	1.61
10b	98.2	96.5	47.3	41.5*	(1.2)	1.61
11a	98.9	90.7	33.8	27.2*	(3.6)	1.54
11b	98.4	95.1	78.9	69.1*	(2.1)	1.50
12 (*in vivo*)	98.2	93.7	5.2	3.3	1.9	1.58
12 (*in vitro*)	97.8	93.7	30.7	25.3*	(1.6)	1.58
13(1)	96.6	91.4	5.2	1.4	3.8	1.58
1	101	98.0	11.2	7.7	3.5	1.68
3	97.2	93.4	24.0	4.7	19.3	1.50
4	96.5	93.8	7.6	2.8	4.8	1.61
14	89.0	85.4	18.2	7.6	10.6	1.61
15	100	95.8	8.1	1.3	6.8	1.58
13(2)	94.4	90.6	12.0	8.8	3.2	1.58
2x	79.6	68.0	59.0	41.5		
2y	89.1	75.6	56.4	36.8		
16†	93.2	88.5	9.2	7.4	1.8	1.64
			Normal sheep			
	95.6	80.6	3.6	1.9	1.7	1.45

Columns 2 through 5 give the per cent solubility of radioiodine at the indicated concentrations of KH_2PO_4-K_2HPO_4. Column 6 is the difference between columns 4 and 5. Column 7 gives the molar concentration of KH_2PO_4-K_2PHO_4 at which the salting out curve of thyroglobulin had its maximum slope.

* These preparations were labeled *in vitro* and were not dialyzed prior to salting out. The value for S-1 in parenthesis has been corrected for iodide (cf. text).

† The preparation employed for salting-out contained .877 mg. of lyophylized tissue extract, 8 ml. of 0.15 *M* NaCl, and 16 ml. of water. Serum was not added.

which would salt out over the same range of buffer concentration presumably as a result of interaction with serum albumin. From experiments performed earlier (3), it was known that approximately 10% of iodide I^{131} added to normal serum precipitates between 1.89 and 2.98 *M* phosphate buffer.

In normal thyroids, S-1 iodoprotein comprised from 1.1 to 6.7% of the soluble iodoprotein (mean 3.5%). Most of the values for the abnormal tissues fell within the normal range. In case 7, it may have been lower, and in the adenomas from cases 3 and 14 it was higher than in normal tissue. In the carcinoma from case 2, a value is not given since the entire salting-out curve differed from those obtained with all other tissues (cf. below).

In the normal and adenoma tissue from case 4, the saline extracts were fractionated with phosphate buffer as indicated in Table 3. These data should be considered in the light of recovery experiments which showed that when whole extracts of the normal and adenoma tissues were carried through the dialysis and lyophylization steps used in preparation of these fractions, the recovery of radioiodine was 70 and 75%, respectively. From the data in Table 3, it is apparent that the adenoma contained much less thyroglobulin (1.4–1.75 *M* fraction) than did the normal thyroid on a weight basis. The distribution of radioiodine in the two tissues was similar, although there was a higher recovery of I^{131} in the 1.92–2.98 fraction (S-1) in the adenoma in keeping with the findings on salting-out curves prepared from the same tissue extracts (Table 2). As discussed in the previous paper (1), each fraction was prepared by 1 or 2 cuts at the concentrations indicated so that contamination of the S-1 fraction with thyroglobulin was expected.

TABLE 3. FRACTIONATION OF SOLUBLE IODOPROTEINS IN HUMAN AND SHEEP THYROID TISSUE

Thyroid tissue	Phosphate fraction	% of total tissue wt.	% of total extract I^{131}*	Thyroglobulin†
				(% of I^{131} in fraction)
Normal-Case 4	<1.4 M	0.5	3.6	
	1.4 –1.75 M	3.4	61.	
	1.92–2.98 M	0.3	0.4	32
Adenoma-Case 4	<1.4 M	0.5	8.5	
	1.4 –1.75 M	0.6	49.	
	1.92–2.98 M	0.4	2.5	23
Normal sheep	<1.33 M	0.3	0.6	
	1.4 –1.75 M	7.9	41.	
	1.75–2.98 M	0.2	1.3	24

* The tissue extract was dialyzed prior to fractionation. "Total" refers only to non-dialyzable radioiodine.
† Determined from salting out curve.

Comparisons of the thyroglobulin fraction and the fraction more soluble than thyroglobulin are presented in Figures 1, 2 and 3. The data for the adenoma are shown since there was less contamination of the S-1 iodoprotein with thyroglobulin, but the findings were similar in the normal tissue.

In Figure 1, salting-out curves demonstrate that the 1.92–2.98 *M* fraction, except for apparent contamination with thyroglobulin, was much more soluble than thyroglobulin. The thyroglobulin in the 1.4 to 1.75 *M* fraction, on the other hand, had been freed of S-1 iodoprotein.

In Figure 2, the sedimentation of S-1 iodoprotein in the preparative ultracentrifuge is shown to be much slower than that of thyroglobulin. The small amount of radioiodine in the 1.92–2.98 *M* fraction at the bottom of the tube (13% of the total) is apparently due to contaminating thyroglobulin, and suggest that the amount of contamination estimated from the salting-out curve (Fig. 1, Table 3) may be overestimated. In another ultracentifuge experiment, fractions prepared from the soluble iodoproteins of case 3—adenoma were compared directly with human serum albumin (Cohn Fraction V). The sedimentation curve of albumin (determined by ultraviolet absorption at 275 mμ) was closely similar to that of iodoprotein which was soluble in 2.28 *M* phosphate buffer.

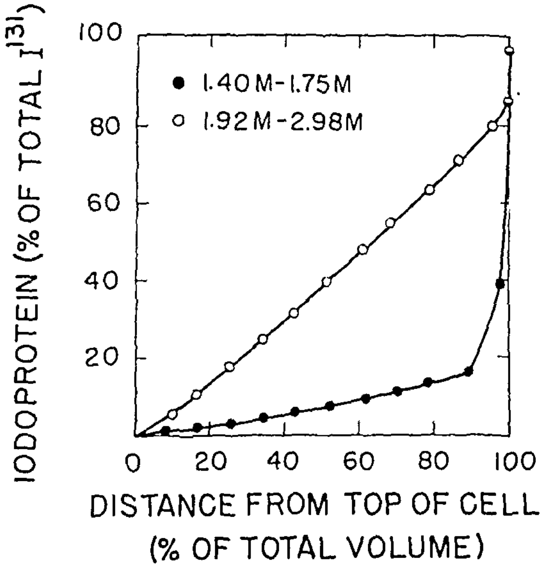

FIG. 2. Sedimentation of soluble iodoprotein fractions (follicular adenoma, case 4) in the preparative ultracentrifuge (39,000 rpm, 3 hours, 0.15 *M* NaCl).

Zone electrophoretic analyses are shown in Figure 3. Whereas thyroglobulin had a mobility like that of alpha globulin in human serum, most of the radioiodine in the 1.92–2.98 *M* fraction had a mobility like that of serum albumin. The remainder of the radioiodine in this fraction was due, at least in part, to contaminating thyroglobulin but there appeared to be a small quantity in the beta globulin zone as well. The presence of serum did not appear to alter the mobility of these fractions. The results of another experiment in which whole saline homogenates of the normal and adenoma tissue in case 3 was used, are shown in Figure 4. The quantity of S-1 iodoprotein in the adenoma was high enough to be apparent in the un-

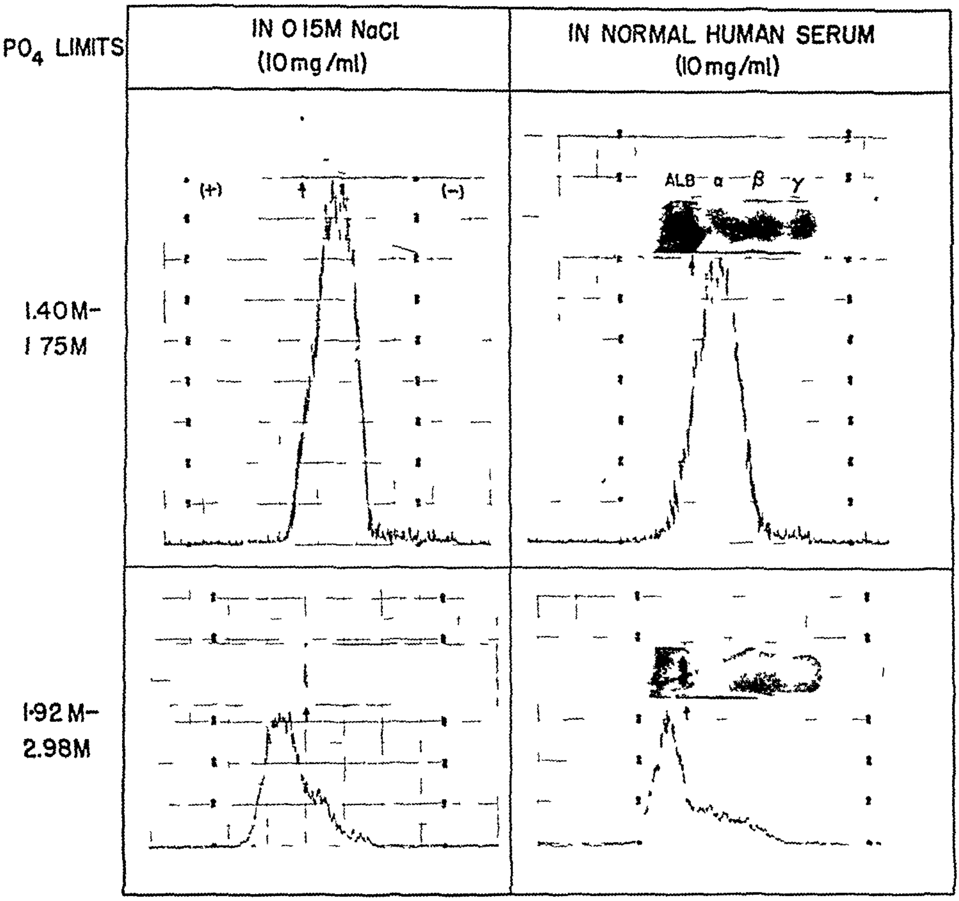

FIG 3. Zone electrophoresis of soluble iodoprotein fractions from the follicular adenoma of case 4 (Barbital buffer, pH 8.6, Γ/2=0.1, reverse flow technique) The lyophilized iodoprotein fractions were dissolved in 0.15 M NaCl or in normal human serum at the concentrations listed The paper strips stained with bromphenol blue are mounted above the corresponding records of radioactivity The arrows indicate the points of application The distance of the baseline portions of each record from the zero line indicates the background counting rate

fractionated material as a radioiodine band with the mobility of albumin. The normal tissue, on the other hand, showed only a thyroglobulin zone. The amount of radioiodine remaining at the origin appeared to be due to insoluble, or particulate, radioiodine in the tissue homogenates.

The electrophoretic mobility of thyroglobulin was examined in 12 subjects. For these analyses, a small quantity of tissue homogenate, supernatant, or extract was added to normal human serum, and the mixture was separated by conventional zone electrophoresis. A typical result is shown by the normal thyroid in Figure 4. The major radioiodine band extended, in all cases, from the anodal end of the beta globulin zone to the anodal end of the albumin zone. The peak of the radioiodine zone varied slightly, as indicated in Table 1, and was either coincident with the alpha-1 globulin

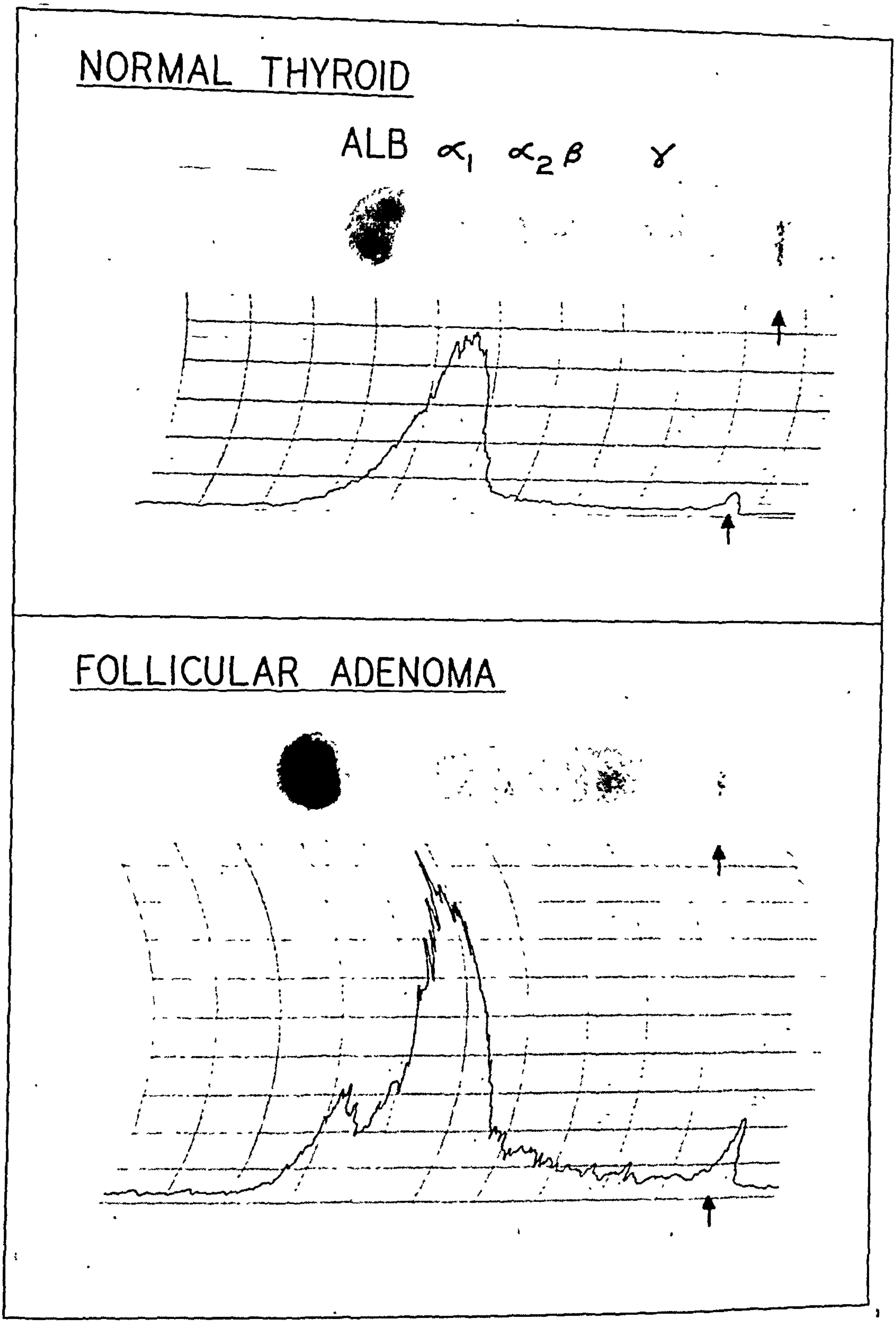

FIG. 4. Zone electrophoresis of unfractionated soluble iodoproteins in the normal thyroid and in the follicular adenoma of case 3. (Barbital buffer, pH 8.6, Γ/2 0.1, conventional technique.) The anode is at the left. Cf. Figure 3.

zone or with the inter-alpha globulin space, or was intermediate between these. The latter is listed in Table 1 as "slow α-1." No consistent difference was found between the normal and abnormal tissue groups, but in normal and abnormal tissue from the same subject, minor differences were sometimes seen (cf. Fig. 4). There was no difference between thyroglobulin labeled *in vivo* and *in vitro*. In about one-half of these experiments, sufficient thyroglobulin was present to be visible on the stained protein strip. It appeared to extend from the inter-alpha space to the anodal end of the alpha-1 zone, and sometimes obscured the alpha-globulin landmarks.

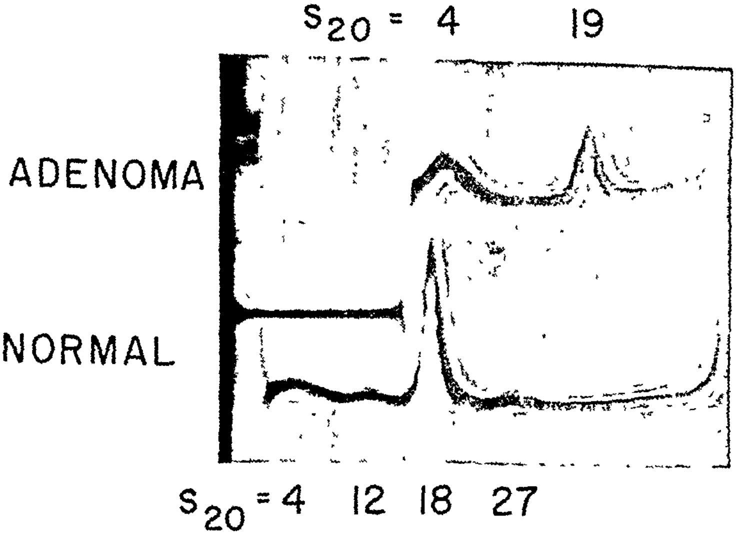

FIG. 5. Sedimentation of the unfractionated saline extracts of the normal thyroid and follicular adenoma of case 4 (59,900 rpm, 0.4% solution in 0.15 *M* NaCl). The extracts had been dialyzed, lyophylized and redissolved in the saline. The values above and below the schlieren patterns give the sedimentation coefficients, in Svedbergs, of the corresponding boundaries. Note the difference in location of the menisci in the standard and "wedge" cells.

Analytical ultracentrifuge analyses of the saline extracts of both normal and adenoma from case 4, and the fractions prepared from these extracts, are presented in Figures 5 and 6 and summarized in Table 4. In keeping with the data in Table 3, the whole extract from the adenoma contained relatively less thyroglobulin ($s_{20} = 19$ S) than did that from the normal tissue. The 1.4–1.75 *M* and the 1.92–2.98 *M* fractions, however, were quite similar in the two tissues. The slow component in each of the more soluble fractions did not break up during centrifugation for 60 minutes but its asymmetry indicated heterogeneity. As discussed elsewhere (1), an identity between the slow component in the schlieren pattern and the S-1 iodoprotein has not been established.

The fractions less soluble than thyroglobulin (Table 3) were not examwas possible to compare the various fractions. Each of the hydrolysates contained iodinated tyrosines, thus attesting to the protein nature of the materials under study. The low yields of iodinated thyronines was expected since all but one of the tissues were labeled *in vitro*. Not only does *in vitro* labeling appear to be a relatively slow process, but it is furthermore limited to 4 or 5 hours. Most of the hydrolysates contained unidentified radioactive components, as was the case in the rat (1). One of these is listed in the Table, and its relationship to monoiodotyrosine has been discussed elsewhere (1).

Comparison of the hydrolysates of particulate iodoprotein with iodoproteins soluble in 0.15 *M* NaCl reveals certain differences. The particulate iodoprotein gave rise to relatively more iodide and to more immobile radioiodine. The latter may be unhydrolyzed or partially hydrolyzed protein. Except in one case, this component was not as large as in the rat tumors (1). The origin of the iodide is unclear. Of the remaining hydrolytic products, the relative proportions of diiodotyrosine (D) and monoiodotyrosine (M) were similar in 3 of the hydrolysate pairs. In the other 3, the ratio D/M was lower in the particulate iodoprotein. The low yield of thyroxine and triiodothyronine made the iodothyronine/iodotyrosine ratio somewhat unreliable. In case 6, however, the thyroxine content of particulate radioiodine was clearly lower.

TABLE 5. HYDROLYSIS OF IODOPROTEINS IN HUMAN AND SHEEP THYROID TISSUE

Case No.	O	I	M	D	T_4	$\frac{D}{M}$	$\frac{T_4+T}{D+M}$	P	I	M	D	T_4	U	$\frac{D}{M}$	$\frac{T_4+T_3}{M}$
	A. Soluble in 0.15 M NaCl							*B. Particulate*							
Normal human															
4	4	3	64	32	2	.50	.021	10	9	61	28	2		.46	.022
5	8	7	48	40	4	.83	.045	4	16	59	12			.20	0
6	5	7	30	58	11	1.9	.12	33	18	6	69		25	2.2	0
Abnormal human															
13(1)	2	5	60	32	2	.53	.022	10	14	61	30			.49	0
4	4	4	55	36	5	.65	.055	8	8	60	27	5		.45	.058
13(2)	2	10	81	16		.16	0	5	17	95				0	0
	C. Insoluble in 1.92 M phosphate							*D. Soluble in 1.92 M phosphate*							
Normal human															
4	8	4	64	31	3	.48	.031	8	9	81	16			.20	0
5	6	3	55	42	2	.76	.021	2	0	39	16		39	.21	0
Abnormal human															
4	3	5	52	34	9*	.65	.10	9	7	75	18	4		.24	.043
Normal sheep	16	15	65	22	3	.34	.015	10	60	50	17			.34	0

Definition of symbols: O = I^{131} at chromatogram origin, I = iodide, M = monoiodotyrosine, D = diiodotyrosine T_4 = thyroxine, T_3 = triiodothyronine, U = unknown (cf text). Values for O and I are in terms of per cent of total iodoprotein. Values for M, D, T_4 and U are in terms of per cent of hydrolytic products other than iodide and immobile material. Deviation of the sum of these values from 100% is due to other unknowns.

Part A = whole thyroid extract or supernatant. Parts C and D = fractions obtained from the extract or supernatant. In all but case 6, the extract or supernatant was dialyzed prior to hydrolysis.

* This hydrolysate also contained 2% T_3.

Comparison of thyroglobulin and the S-1 iodoprotein (parts C and D of Table 5) reveals a consistently lower diiodotyrosine and thyronine content in the latter except in the sheep thyroid. The reason for the unusually large amount of iodide in sheep thyroid S-1 is unknown.

As discussed previously (1) the difference in hydrolysis products among the iodoproteins indicate that the proteins are not artifacts of the fractionation procedures.

COMMENTS

The iodoproteins of human and sheep thyroid tissue described in this paper are analogous to those found in a functioning rat thyroid tumor (1). As proposed in the preceding paper, they may be classified according to their solubility in 0.15 *M* NaCl as particulate iodoprotein, or "thyroid P-1" and soluble iodoproteins, or "thyroid S-1" and thyroglobulin.

The association of human thyroid P-1 iodoprotein with specific subcellular particulate fractions has not been as well delineated as it has in the rat. Even in the rat, however, its apparent association with cell nuclei has not been conclusively proved. The quantity of thyroid P-1 iodoprotein was highest in a follicular thyroid carcinoma, but was apparently present in normal thyroids and in benign goiters as well. Particulate iodoprotein was not studied in the sheep.

The soluble thyroid iodoproteins of man and sheep were different in certain respects from those found in the rat. The solubility of human thyroglobulin in phosphate buffer was slightly greater than that of rat thyroglobulin, and sheep thyroglobulin appeared to be less soluble than either human or rat. These thyroglobulins also differed in electrophoretic mobility, those of man and sheep being slower than rat thyroglobulin. The slight differences in electrophoretic mobility of human thyroglobulin among the tissues studied are like that described in one case by Easty, Slater and Stanley (4).

Thyroid S-1 iodoproteins were different in each of the species studied, as revealed by zone electrophoresis. In man, S-1 iodoprotein had the mobility of serum albumin, although there may have been a small amount moving with the serum globulin as well. In the sheep, S-1 iodoprotein appeared to consist of 2 main components, one with the mobility of human albumin, and one which moved with human alpha globulin. In the rat, S-1 iodoprotein was composed of 3 more or less equal components, the fastest of which moved behind human serum albumin.

It is of considerable interest that human thyroid S-1 iodoprotein bears a close resemblance to the iodoprotein found in the blood of patients with functional carcinoma (5, 6) as well as that in a patient with congenital goiter (7) and, although the data are less complete, in chronic thyroiditis (8). The data reported here demonstrate that thyroid S-1 iodoprotein is present in normal thyroid glands, as well as in tissue representing a variety of benign and malignant thyroid disorders. The highest proportion of this material was found in 2 follicular adenomas. It has not been shown, however, whether this is the result of an absolute increase in the concentration of S-1 iodoprotein, or a relative decrease in thyroglobulin.

Additional evidence that thyroid S-1 iodoprotein is related to the iodoprotein in blood is the finding that rat thyroid S-1, while differing from thyroid S-1 in other species, resembles the iodoprotein found in the blood of rats with thyroid tumors (1).

Further speculations on the nature of the non-thyroglobulin iodoproteins and their possible significance are discussed in the preceding paper.

Acknowledgments

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THE EFFECTS OF HYPOTHALAMIC LESIONS ON GOITROGENESIS AND PITUITARY TSH SECRETION IN THE PROPYLTHIOURACIL-TREATED GUINEA PIG[1]

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ABSTRACT

Propylthiouracil (PTU)-induced goitrogenesis was selectively impaired in male guinea pigs by anterior hypothalamic lesions. These lesions also reduced or abolished the PTU-induced enlargement of the adenohypophysis and the increase of thyrotrophin (TSH) stores therein, without causing gonad atrophy. More posterior lesions affected TSH secretion less and did cause gonad atrophy. The findings are interpreted to mean that neither the synthesis nor the release of TSH can reach maximal levels in the absence of the neural structures which were damaged.

IN RATS, appropriate hypothalamic lesions have been found to impair, but not abolish, thyrotrophin (TSH) secretion (1–5). In dogs, on the other hand, similar lesions seem to eliminate TSH secretion, or at least its release phase, since they reduce both thyroid size and I^{131} uptake to hypophysectomy-like levels (6). This apparent species difference in the extent to which TSH secretion may be decreased by hypothalamic damage suggests that further comparative studies, such as the one presented here, may be of value. In this investigation the question of whether not only the *release* but also the *synthesis* of TSH can be impaired by lesions of the hypothalamus has been approached through the study of pituitary TSH stores and thyroid activity in the propylthiouracil (PTU)-treated guinea pig. The choice of experimental animal was governed by the following considerations.

The amount of hormone synthesized during an experimental period must equal the algebraic sum of the amounts stored in, and released from, the adenohypophysis. The stored TSH may be assayed directly, but indices of released TSH, such as various thyroid responses or even measurements of circulating TSH levels, are only indirect since they give no indication of how much released TSH may have been cleared or destroyed by extrathyroid mechanisms. In the rat, the effects of hypothalamic lesions are made most evident by chronic PTU-treatment (1–3, 5). However, the pituitary TSH stores of PTU-treated rats are low, despite a rapid rate of TSH synthesis (7). Because of the uncertainty with which release can be

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estimated, the effects of hypothalamic lesions on blood and pituitary TSH levels in such animals (5) may be difficult to interpret. In the guinea pig, on the other hand, chronic PTU-treatment leads to an increase of pituitary TSH stores as well as thyroid enlargement (7, 8). Thus, all 3 phases of TSH secretion—synthesis, storage and release—proceed at accelerated rates during goitrogenesis in this species. In such a situation, the impairment of TSH release by hypothalamic lesions should effect a decrease of thyroid hypertrophy, whereas a lessened capacity for TSH synthesis should tend to diminish adenohypophyseal TSH accumulation.

MATERIALS AND METHODS

Hypothalamic lesions were made, under ether anesthesia, in young (212–370 gm.) and adult (400–650 gm.) male guinea pigs, using a "homemade" stereotaxic device.[2] A direct (anodal) current of 3 to 4½ m.a., applied for 20–45 seconds, was used for each lesion. Coordinates were determined empirically. In each experimental animal 2 or 3 lesions were placed so as to fuse across the midline. Most lesions were made in the anterior hypothalamus, but a few were placed in the median eminence region and some in the anterior thalmus. Sham operations consisted of trephining the skull, with or without electrode insertion.

Postoperatively, the guinea pigs were placed in either wire mesh cages (6–8 young) or wooden pens (7–12 adult). Since no behavioral effects of the lesions were noted, experimental and control animals shared the same quarters. The basic diet was Purina fox chow meal, mixed with PTU[3] (0.15%). Raw vegetable supplements were fed 4–7 times weekly, and tap water was allowed *ad libitum*. Flaked oats, ascorbic acid (2%) and sulfadiazene (2%) with $NaHCO_3$ (6%) were added to the basic diet of the adults (Groups VII and VIII). The sulfadiazene was found to decrease the incidence of respiratory infections. Both young and adult "euthyroid" controls (unoperated) received similar treatment, but without either PTU or sulfadiazene-bicarbonate.

The guinea pigs were fed the PTU diet for 68–113 days, and weighed biweekly. At sacrifice, thyroid-serum radioiodide concentration ratios (T/S) were measured as follows. One hour after a subcutaneous injection of 30 mg. of PTU (in alkaline solution), 20 μc. of NaI^{131} were injected subcutaneously into each animal. Blood samples were removed 60–80 minutes later under ether anesthesia. The guinea pigs were then killed and the pituitary and thyroid glands removed immediately. The radioactivity in 50 mg. of serum and in about 10 mg. of thyroid tissue was measured by β-counting. T/S ratios were calculated conventionally (9).

Adenohypophyses, stripped of neural lobes, were weighed rapidly and frozen (in 0.5 ml. of 0.25% acetic acid-0.85% NaCl solution) for subsequent TSH assay. Brains were fixed in 10% neutral formalin, Tissuemat-embedded, and sectioned serially at 10μ. Every 9th and 10th section was stained with thionine for lesion-localization studies. Thyroid, testis and seminal vesicle weights were recorded and the tissues processed for histologic study. The organ weights were converted to relative weights (mg./100 gm. body weight at sacrifice) and are discussed as such in this paper.

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[2] The stereotaxic device utilized a cat-monkey headholder. The ear bars were 2⅝″ high, their support posts were 3″ apart and the tooth clamp was adjustable. (This headholder, mounted on a 7″ square base, was supplied by the late Mr. Roy Johnson.) A raised (2″) level platform (5″×2″) was mounted on the base plate to the left of the headholder, and the electrode-carrier unit of a Kreig-Johnson rat stereotaxic instrument bolted thereon with #210 thumb bolts. This improvised device allowed the same stereotaxic instrument to be rapidly converted for use on either rats or guinea pigs. A unipolar electrode, protruding 15–17 mm., with 1 to 1½ mm. bare at the tip, was used for the guinea pigs.

[3] The PTU was generously donated by Dr. R. J. Turner of the American Cyanamid Company.

TSH bioassays were carried out in stasis tadpoles on homogenates of 5–10 pooled anterior lobes, as previously described (10).

RESULTS

Goitrogenesis. PTU treatment for 68–75 days increased the mean thyroid weight of 12 young guinea pigs without hypothalamic lesions approximately four-fold (Table 1, Group II vs. Group I, P ≠.004). The same treatment raised thyroid weight less than two-fold in 15 animals with such lesions (Group III vs. Group I, P ≠.001). The lesions thus decreased goitrogenesis by more than 50% (Group III vs. Group II, P ≠.02). However, since some of the PTU-controls did not show appreciable thyroid enlargement, it was not possible to attribute the absence of goiter in individual animals of Group III to their lesions, even though "goiter block" was evident in the group as a whole. To raise control thyroid weights further, so that individual goiter-blocking lesions might be selected, a longer PTU-treatment period (>100 days) was used subsequently. For 5 young PTU-controls (Group IV), the longer treatment resulted in striking (ten-fold, P <.001) thyroid enlargement. Similar treatment produced less marked, but still significant (P ≠.001), goiter formation in 5 older controls (Group VII). Fifteen younger (Groups V and VI) and six older (Group VIII) guinea

TABLE 1. BODY AND ORGAN WEIGHTS AND T/S RATIOS IN "EUTHYROID" CONTROLS AND PROPYLTHIOURACIL-TREATED GUINEA PIGS, WITH AND WITHOUT HYPOTHALAMIC LESIONS

Group		Body weight (gm.)	Days on PTU	Wt. gain* (gm./day)	Hypophysis†	Organ weights—mg./100 gm.		Thyroid	T/S
						Testis	Seminal vesicles		
I	Euthyroid Control (13)‡	584±15§	0	—	1.70±.09	527±28	119±6	9.6±0.7	57±8
II	PTU Control (12)	461±17	68 to 75	1.88±.30	2.48±.18	550±25	112±9	43.9±10.4	317±72
III	Hypothal Lesions (15)	444±29		1.86±.31	1.99±.11	594±27	178±12	17.5±1.8	369±93‖
IV	PTU Control (5)	553±33	102 to 113	2.30±.10	3.04±.38	537±18	164±27	96.4±9.8	198±5
V	"Goiter Block" (6)	597±49		2.30±.42	1.65±.18	585±39	176±29	19.0±2.5	271±37
VI	"Ineffective" Lesions (6)	512±14		2.03±.23	1.95±.27	429±39	122±25	74±19.4	250±45
VII	PTU Control (5)	1071±80	103	2.80±.52	1.70±.26	355±18	149±17	43.7±5.5	486±54
VIII	"Ineffective" Lesions (6)	938±95		2.35±.68	1.26±.11	460±35	187±7	39.7±6.7	581±133

* During PTU feeding.
† Anterior lobe only.
‡ No. of animals.
§ S.E. of mean.
‖ Average of only 11 data.

pigs with hypothalamic lesions survived the prolonged PTU regimen. Of the younger, 3 which exhibited severe gonadal atrophy were not included in the table (but are included in Fig. 1); the remainder were divided into "goiter block" (V) and "ineffective lesion" (VI) groups on the basis of thyroid weight. Group VIII did not exhibit "goiter block," although one of its members had a thyroid weight in the "goiter block" range (21.2 mg./100 gm.).

T/S ratio. The average "euthyroid" T/S ratio was 57 ±8. PTU treatment increased this value from four- to ten-fold (Table 1). Neither in Group V nor in other groups did lesions prevent this increase consistently, although a few animals in Group III did have T/S ratios in the "euthyroid" range. The findings even suggest that the lesions tended to increase the T/S ratios, but the average increases were not statistically significant. Thyroid size and T/S ratios tended to be related inversely (Fig. 1).

Thyroid microscopic structure. PTU-treatment for 68–75 days induced

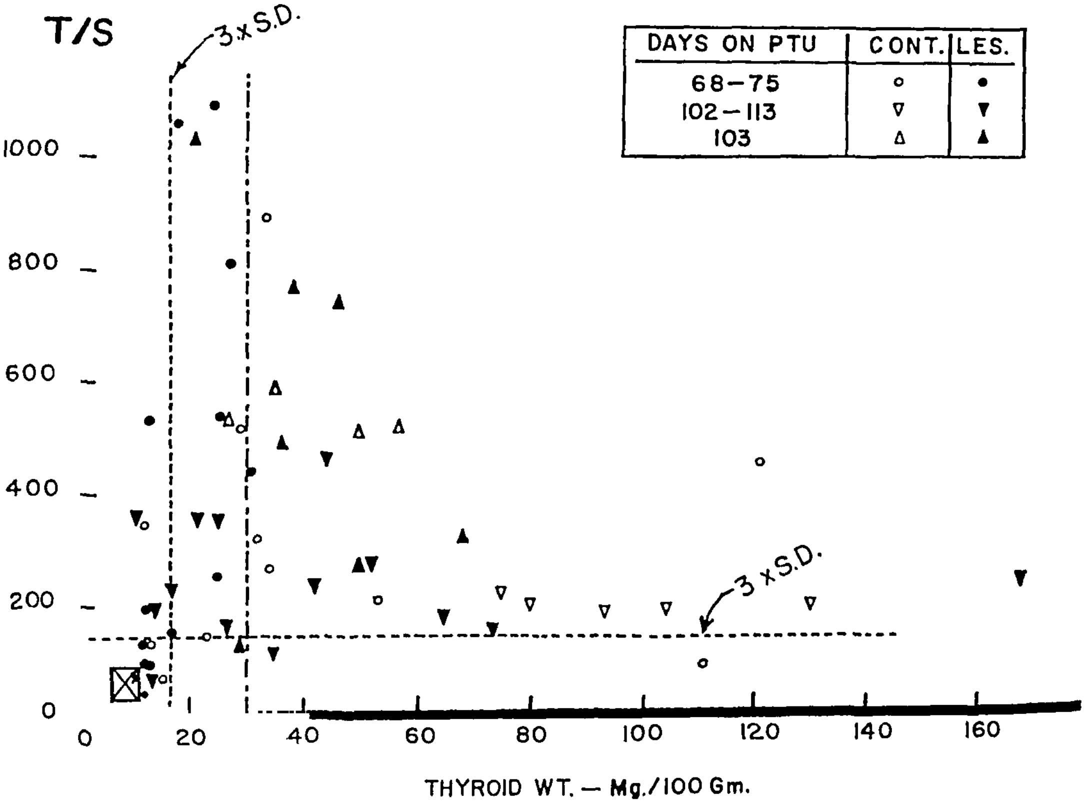
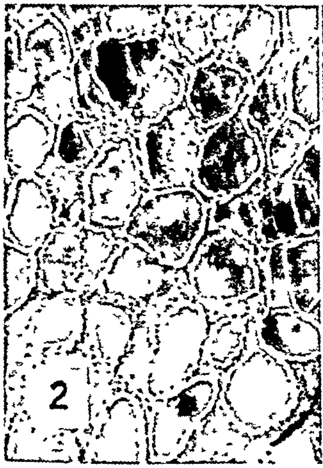
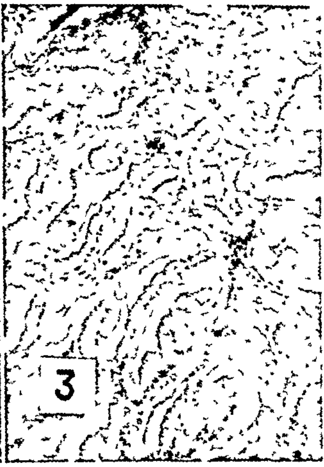


FIG. 1. Graph of thyroid weight and T/S ratio for individual male guinea pigs fed propylthiouracil (PTU) for 68–113 days. The small square with crossed diagonals at the lower left of the graph indicates the mean thyroid weight and T/S ratio (center of square) and standard deviation (perimeter of square) for 13 "euthyroid" controls (Group I, Table 1). The dotted lines which enclose this square represent 3 times the standard deviation and should limit over 99.9% of individual "euthyroid" values. The vertical line made of dots and dashes indicates the arbitrary limit (30 mg./100 g.), above which "goiter block" was not considered to be appreciable. Open symbols = PTU controls (see legend); solid symbols = hypothalamic lesions.

marked changes in the microscopic thyroid structure of controls (Group II), which roughly paralleled in extent the increases of thyroid weight. "Euthyroid" (Group I) glands were characterized by squamous acinar epithelium and dense colloid containing only small peripheral vacuoles (Fig. 2). Three (25%) of the Group II glands, weighing between 12 and 16 mg./100 gm., closely resembled those of Group I; the others had cuboidal or columnar epithelium and whatever colloid they contained was more dilute. In 9 (60%) of the Group III guinea pigs thyroid structure appeared inactive. In general, as thyroid weight increased, cell height did too and the colloid became progressively more dilute and highly vacuolated. The largest glands were virtually devoid of colloid and their follicles appeared collapsed.





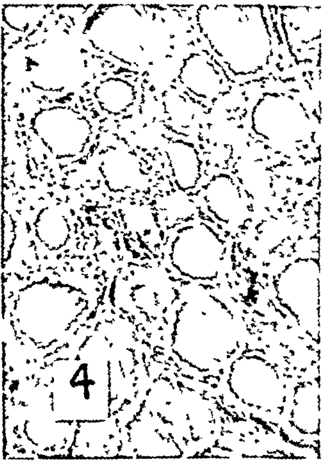


FIG. 2. Thyroid gland of guinea pig not given PTU. Squamous acinar epithelium, dense colloid.

FIG. 3. Thyroid gland of guinea pig fed PTU for 113 days. Weight: 104 mg./100 gm.

FIG. 4. Thyroid gland of guinea pig with "goiter block" after 113 days of PTU treatment. Weight 11.4 mg./100 gm.

The thyroid glands of 102–113 day PTU controls (Group IV) similarly looked highly stimulated (Fig. 3). In Group V, only the largest gland exhibited cuboidal epithelium and dilute colloid. The others had squamous epithelium and colloid which seemed to be only a bit less dense (Fig. 4) than that seen in Group I.

The two smallest glands in Group VI (42 and 52 mg./100 gm.) appeared less stimulated than the rest which resembled those of group IV. However, even these two glands had low columnar epithelium, definitely greater in height than any in Group V.

Pituitary size and TSH content. In the younger controls, PTU treatment for 102–113 days increased anterior pituitary weight 46% (Group IV vs. Group I, P = .001). Both Group V and Group VI lesions reduced this hyper-

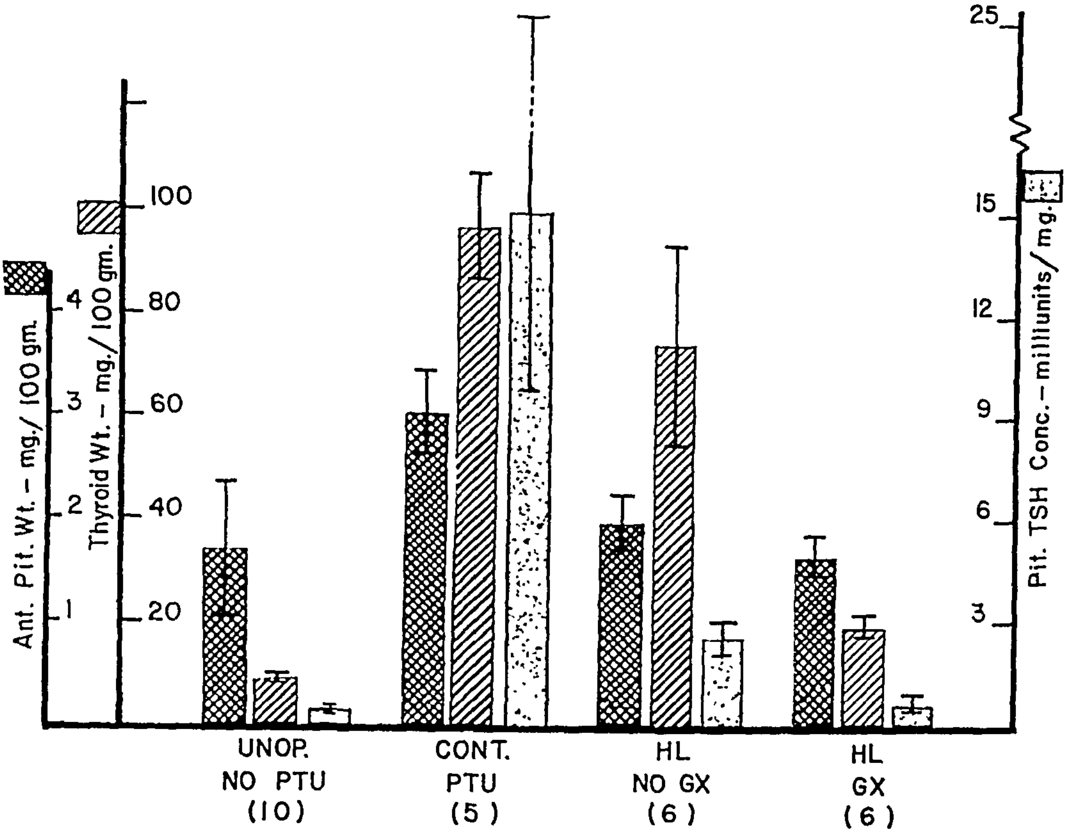
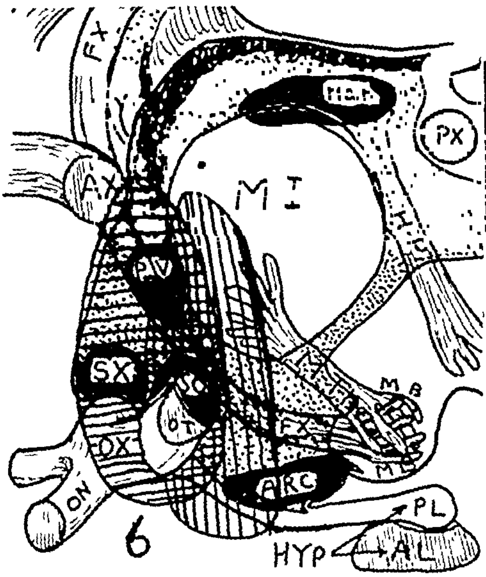


FIG. 5. Chart of mean thyroid and adenohypophyseal weights and adenohypophyseal TSH concentrations for Groups I, IV, VI and V (see Table 1). Vertical lines through tops of bars represent either standard error of mean (for gland weights) or range corresponding to 1 standard error of the mean tadpole thyroid cell height response (for anterior pituitary TSH bioassays).

trophy appreciably (Table 1), but the effects were not statistically significant. Adenohypophyseal weight was also greater in the older PTU controls (Group VII) than in comparable guinea pigs with lesions (Group VIII), but as there were no "euthyroid" controls of similar age and body weight, this difference cannot be evaluated fully.

In Figure 5 the relative thyroid and anterior pituitary weights for Groups I, IV, VI and V of Table 1 are compared with the corresponding adenohypophyseal TSH concentrations. These were 0.45 (0.35–0.55), 15.0 (10–25), 2.5 (1.8–3.0), and 0.6 (.45–.80) milliunits (range) per mg. of wet weight, respectively. The concomitant changes of anterior pituitary size and TSH concentration, and of thyroid size, in guinea pigs receiving chronic PTU-treatment are in accord with previous findings (7, 8). The lesions of Groups V and VI either decreased or abolished these changes. Although the anterior lobe hypertrophy (75%) seen in Group IV was not as great as the increases (>1000%) in thyroid size and anterior pituitary TSH concentration, it should be taken into account in evaluating total TSH stores. These were calculated (mean TSH concentration × mean



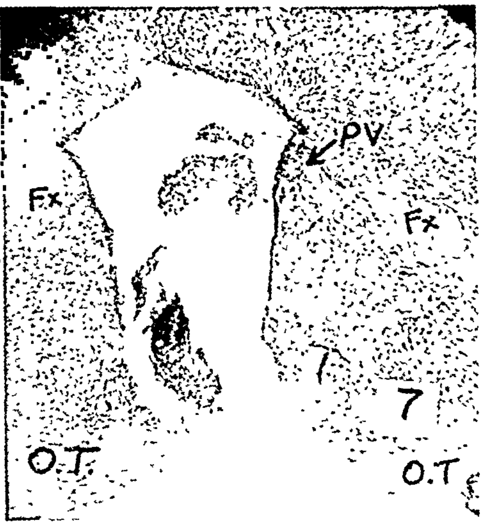


FIG. 6. Diagram of sagittal section of guinea pig diencephalon, with parasagittal structures shown by projection. ON, OX, OT = optic nerve, chiasm and tract; AX and PX = anterior and posterior commissures; MB = mammillary body; MI = massa intermedia; FX = fornix; M-Th = mammillo-thalamic tract; H-P = habenulo-peduncular tract; Hab. = habenular nucleus; P-V = paraventricular nucleus; S-X, S-O = suprachiasmatic and supraoptic nuclei; Arc = arcuate nucleus; Hyp. = hypophysis (P.L. = posterior lobe, A.L. = anterior lobe); Third ventricle is stippled. The lesions of group V were in the area shown by horizontal hatching. Group VI lesions were in the area shown by vertical hatching.

FIG. 7. Section through a Group V lesion (guinea pig 50, thyroid weight 11.4 mg./100 gm.; testis and seminal vesicle weights were 715 and 185 mg./100 gm., respectively). The lesion did not involve the median eminence FX = fornix; OT = optic tract.

adenohypophyseal weight) to be about 4.3, 250, 25 and 5.6 U.S.P. milliunits of TSH *per gland* for Groups I, IV, VI and V, respectively.

Gonads. Three lesion-bearing guinea pigs with marked testicular and seminal vesicle atrophy as well as "goiter block" were not included in Table 1. Slight testicular atrophy was seen in 5 of the 6 animals of Group VI.

The gonads of the other animals were well maintained. In fact, seminal vesicle weight was increased significantly ($P < .001$) in Group III, suggesting heightened androgen secretion. This accessory sex gland hypertrophy, cited elsewhere (11), was observed less frequently in the other groups.

Lesion localization. In the 6 animals of Group V, the fairly symmetrical lesions destroyed the region of the paraventricular nuclei and extended between the columns of the fornix from just behind the anterior commissure well into the anterior part of the ventricle (Fig. 7, cf. Fig. 6). They usually reached to the floor of the brain at some point between the optic chiasm and the median eminence.

Similar lesions were found in Group III animals without goiters. Although it cannot safely be said that they caused "goiter block," it is possible to state that no guinea pig with such a lesion exhibited a large goiter.

In general, the "ineffective" lesions were somewhat more posterior (Group VI) (Fig. 6) or else similarly placed but smaller or asymmetrical (Group VIII). Those which caused gonad atrophy were basal and greatly damaged either the infundibulum (2 of the 3 omitted from Table 1) or the arcuate nucleus region. In this connection it is of interest that the testes and seminal vesicles of 5 of the 6 guinea pigs in group VI weighed less than the control (Group IV) average. The lesion leading to gonad atrophy in the male guinea pig thus appears to be similar to that causing gonad atrophy in the male rat (12).

DISCUSSION

These findings clearly indicate that appropriate hypothalamic lesions may produce "goiter block" in the guinea pig, preventing not only most of the thyroid weight increase but also, to a great extent, the microscopic manifestations of goitrogenesis. On the other hand, few lesions prevented an increase of the T/S ratio after PTU treatment and in no instance was this ratio significantly less than the mean ratio for "euthyroid" controls (Fig. 1). Although data are not available on the T/S ratios of PTU-treated, hypophysectomized guinea pigs, it seems reasonable to assume that in such animals the T/S ratio would be less than 57. Taken together with the fact that most of the lesions which affected thyroid structure did not prevent the PTU-induced rise of the T/S ratio (Fig. 2), this seems to indicate that the lesions did not block TSH secretion completely. The T/S ratio in the rat responds to very much smaller amounts of exogenous (13) or endogenous (14) TSH than are needed for thyroid hypertrophy or hyperplasia. If these parameters can be assumed to respond similarly in the guinea pig, it follows that the extent to which TSH release in this species has been impaired by hypothalamic lesions is greater than in the rat (4, 5) but less than in the dog (6).

The "euthyroid" T/S ratio (57 ±8) found in this study was somewhat higher than that found in rats not treated chronically with goitrogen (4, 9). This is of interest in view of the relatively low level of thyroid activity in the normal guinea pig (8).

The marked reduction of TSH stores in the adenohypophyses of PTU-treated guinea pigs with hypothalamic lesions, whether or not they developed goiters, suggests that TSH synthesis may have been impaired even more than release. This point will require further investigation, however. Since thyroid size is only a crude index of TSH release, there might well have been some degree of "goiter block" even in animals with considerable thyroid enlargement. The determination of circulating TSH levels might be helpful in elucidating this question. In any case it is clear that both the release and storage of thyrotrophin were decreased markedly by lesions which did not impair gonad maintenance. We interpret this to mean that such lesions can selectively impair TSH *synthesis*, as well as *release*.

This seems to be in contrast with the failure of hypothalamic lesions to prevent TSH reaccumulation by the adenohypophyses of rats from which PTU had been withdrawn (15, 16). Although TSH synthesis in the rat did not appear to be influenced by hypothalamic lesions in this situation, it clearly was impaired under other conditions (5). In the absence of any report on the effects of hypothalamic lesions on the TSH "rebound" phenomenon in the guinea pig, further comparison of the neural influences on TSH synthesis in these two species must be withheld.

It is not yet possible to identify the neural structures which appear necessary for augmented TSH secretion in the guinea pig. As in the rat (1–4, 12) and dog (6) they seem to lie, at least in part, in the anterior hypothalamus. They may be damaged demonstrably by lesions which do not seem to interfere with testicular maintenance and therefore must be somewhat separate from the neural mechanisms concerned with gonadotrophin secretion.

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ANTIGOITROGENIC AND CALORIGENIC ACTIVITIES OF THYROXINE ANALOGUES IN RATS[1]

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ABSTRACT

The relative potencies of more than forty thyroxine analogues and a soluble hog thyroglobulin extract were established in parallel goiter-prevention and calorigenic assays against the same thyroxine standard. By both methods of assay twelve of the preparations had activities ranging from 5% to 800% of that of thyroxine. The activities, if any, of the remaining analogues were less than 3% of that of the standard.

Analysis of area-under-curve and speed of response in calorigenic assays indicated that all the active analogues and soluble thyroglobulin were qualitatively similar with respect to this parameter of thyromimetic function in normal adult male rats. Thyroglobulin was characteristically more active than expected when its potency, relative to thyroxine, was calculated on the assumption that its thyronine-bound iodine, as determined by the Blau, 1935, procedure, was in the form of thyroxine.

Correlations of structural changes with corresponding assay activities indicated the following generalizations: (a) analogues having an intact alanine side chain required iodine substituents at least in the 3 and 5 positions as a condition for biological activity. Thyronines having iodine substituents in the 3, the 3 and 3′, or the 3, 3′ and 5′ positions were biologically inactive at relatively high dose levels; (b) the potency of analogues with deaminated side chains decreased as the chain length increased from two to four carbons; (c) analogues having a deaminated side chain required iodine substituents in the 3 and 5 positions with additional iodine substitution in either or both the 3′ and 5′ positions, as a condition for biological activity, but 3,5,3′-triiodo compounds were not necessarily more active than the corresponding 3,5,3′,5′-tetraiodo derivatives; (d) inactive 3,5-diiodothyropropionic acid was activated by introduction of methyl groups in positions 3′ and 5′; (e) replacement of the phenolic hydroxyl group at the 4′ position by the methyoxy group caused a significant reduction in goiter-prevention activity.

Ratios of antigoitrogenic to calorigenic potencies, ranging from approximately 1.0 for 3,5,3′-triiodo-l-thyronine to 5.0 for 3,5,3′,5′-tetraiodothyroacetic acid, were indicative of a real dichotomy of action for some of the analogues.

The decomposition of 3,5,3′,5′-tetraiodothyroacetic acid and two other analogues, in alkaline solution, suggested a note of caution with regard to the interpretation of previously published bioassays for such preparations.

Comparative results showed that in most cases, the calorigenic assay could be used interchangeably with the antigoitrogenic assay to predict the exogenous dose of an analogue equivalent to the daily endogenous hormonal output by normal rat thyroids.

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[1] Presented at the 1958 Meeting of the American Goiter Association.

BIOASSAY results, covering forty-five thyroxine analogues, are presented as a contribution toward the increasing effort to delineate the effects of alterations in molecular structure on biological activity (1–5). The difficulties of correlating structure and activity from the reports of many investigators are enhanced by the variety of bioassay methods employed, unreported activity for a given compound by one or more assay methods, and the uncertainty regarding the identity and purity of some of the tested compounds.

This study was designed to overcome the aforementioned difficulties by employing a closely correlated program of organic synthesis and bioassays. Thus, virtually all the analogues were prepared and carefully characterized in these laboratories. Antigoitrogenic and calorigenic assays employing rats of the same strain and source were conducted in these laboratories while Money *et al.* (6, 7) assayed the compounds for I^{131} uptake activities in rats and also for induction of tadpole metamorphosis. Accordingly, comparisons between analogues, assay methods and species could be made with greater confidence.

During the course of this work an unanticipated decomposition of certain thyroxine analogues in alkaline solution was observed. Special attention was therefore given to the critical evaluation of the results of bioassays performed on such compounds.

MATERIALS AND METHODS

Antigoitrogenic Assay

During the fourteen day test period female albino rats, weighing 160–190 gm., were allowed free access to tap water and powdered rat diet containing thiouracil (0.3% by weight). Room temperature was maintained at 75° ± 3° F. Solutions of thyroxine and one or more analogues[2] were assayed simultaneously. Rats were injected subcutaneously, daily, on Days 0 through 13. Five to ten animals were allotted to each dose level and three or more dose levels were employed for each of the active analogues. On Day 14, the thyroid glands were rapidly removed, cleaned, weighed to the nearest 0.1 mg. and recorded for each rat as mg. of gland weight per 100 gm. of body weight. Preparations suspected of having little or no activity were screened at a single dose level which was at least twenty times the dose of thyroxine (4 μg./rat/day) required to produce a 67% inhibition of thiouracil-goiter. Compounds found active by screening were reassayed at three or more dose levels.

Calorigenic Assay

Male albino rats weighing 280–320 gm. were trained to eat their total daily requirement of powdered rat diet between 11:30 A.M. and 4:30 P.M. daily (8) for at least two weeks prior to and during the BMR test period. BMR tests consisted of three or six consecutive ten-minute readings of oxygen comsumption for each rat on several days before, during and after drug administration. These determinations were always made between 8:00 A.M. and 11:30 A.M. Thus, the fasting period preceding each BMR test varied between sixteen and nineteen hours. Twelve Benedict-type (9) closed circuits maintained at 29° ± 0.2° C made it convenient to run twenty-four to forty-eight rats each morning. The lowest two or four ten-minute readings of the three or six, respectively, which were made for each rat were averaged, corrected to standard conditions and recorded as mg. O_2/Kg. body weight/hour. For the most part, percentage changes in BMR, during and after drug administration, were based on the average pretreatment level for each rat. However, in some assays it was more convenient to use the day to day average of a placebo-injected control group as the basis for calculating the percentage changes of treated groups on the same day. Thyroxine and one or more analogues were assayed concomitantly at two or more dose levels per preparation, using five to twelve rats per dose level. A shortage of one active analogue resulted in tests at a single dose level. The active analogues were injected subcutaneously, once per day, for a period of five to fourteen days. All compounds suspected of having little or no thyroxine-like activity were screened at a single dose level (1 or 2 mg./rat/day/3–4 days, subcutaneously) in groups of four rats per preparation. These dose levels were five to ten times greater than the amount of thyroxine required to produce a strong response.

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[2] Crystalline *l*-thyroxine and 3,5,3′-triiodo-*l*-thyronine were obtained from Cyclo Chemical Corp., Cal. Gifts of the following analogues are gratefully acknowledged: 3,5,3′,5′-tetraiodothyroacrylic acid from Dr. Stanley Wawzonek, State Univ. of Iowa, Iowa City; 3,5-diiodo-3′,5′-dimethylthyro-propionic acid from Dr. Norman Kharasch, Univ. of Southern California, Los Angeles; 3,5,3′,5′-tetrachloro-*dl*-thyronine synthesized by Sir. C. R. Harrington and furnished by Dr. J. Lerman, Boston.

Solutions

In the early phases of this study preparations were dissolved in a minimum volume of 0.1*N* NaOH and diluted to required concentrations with physiological saline. Subsequently, several analogues were prepared for injection by dissolving them in ethyl alcohol with added 0.1*N* NaOH (if needed). Saline (adjusted to pH 8–9) was used for dilutions, and the final concentration of alcohol was always 10% or less. A solvent consisting of ethyl alcohol/propylene glycol, 10/90, V/V, was required for a compound which was extremely labile in the presence of alkali, under certain conditions. Volumes of solution sufficient to last up to fourteen days were distributed in stoppered vials and maintained frozen between injections. Daily doses for each rat were usually contained in a volume of 0.5 cc. to 1.0 cc. In order to minimize contamination of samples in storage vials, hypodermic needles were never used for drawing doses after the needles had been used for injection.

Computation of Results

In assays designed with two or more dose levels, for standard and unknowns, the regression equation, Y = a + b log. X, was calculated for each preparation (10). The several b-values in a completed experiment were replaced by the average slope (b) with due regard for inequalities in numbers of observations and/or dose increments (11). The potency of each unknown (U) relative to standard (S) was calculated as the antilog. of [av. log. Xs − av. log. Xu + (av. Yu − av. Ys/av. b)]. In addition, the resulting equations were used for the derivation of X-intercept values in each method of assay. Such values for goiter-prevention results were first used by Dempsey and Astwood (12) but their usefulness in the analysis of calorigenic assays appear to have been largely overlooked. When a compound was tested at a single dose level, the amount of thyroxine required to produce the same response was read from the concomitant standard curve. This value, divided by the dose of unknown, gave a relative potency estimate for the preparation. The validity and precision of these assay methods, under the experimental conditions described here, were presented and discussed in a previous report by Stasilli and Kroc (13). Confidence in the results is greatly strengthened by the fact that "standard" was always tested concomitantly with each unknown in homogeneous groups of rats. Thus, variations in results which might be ascribed to species, sex, weight or seasonal and environmental differences, etc., (2) were either nullified or greatly diminished.

RESULTS AND DISCUSSION

Active analogues—relation of structure and activity:

Typical calorigenic response curves for thyroxine, some of its analogues and soluble thyroglobulin are shown in Figures 1 and 2. It is apparent that

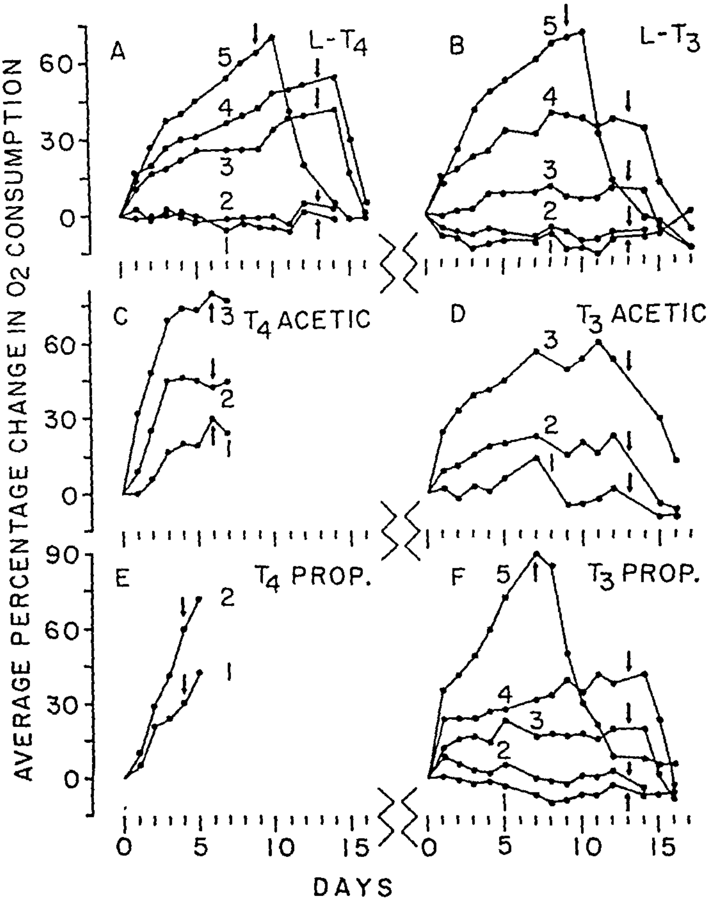


FIG. 1. *BMR response of normal adult male rats during, and after, 5–14 days subcutaneous injections of thyroxine analogues (μg./100 gm. body weight/day):*

A. L-Thyroxine (sodium pentahydrate): 1, alkaline-saline controls; 2, 2 μg.; 3, 6 μg.; 4, 16 μg.; 5, 55 μg.

B. L-Triiodothyronine: 1, alkaline-saline controls; 2, 0.3 μg.; 3, 1 μg.; 4, 3 μg.; 5, 10 μg.

C. 3,5,3′5′-tetraiodothyroacetic acid (in alcohol-propylene glycol): 1, 50 μg.; 2, 150 μg.; 3, 450 μg.

D. 3,5,3′-triiodothyroacetic acid: 1, alkaline-saline controls; 2, 30 μg.; 3, 105 μg.

E. 3,5,3′,5′-tetraiodothyropropionic acid: 1, 420 μg.; 2, 1270 μg.

F. 3,5,3′-triiodothyropropionic acid: 1, alkaline-saline controls; 2, 30 μg.; 3, 90 μg.; 4, 300 μg.; 5, 900 μg.

Last daily injection shown by arrow.

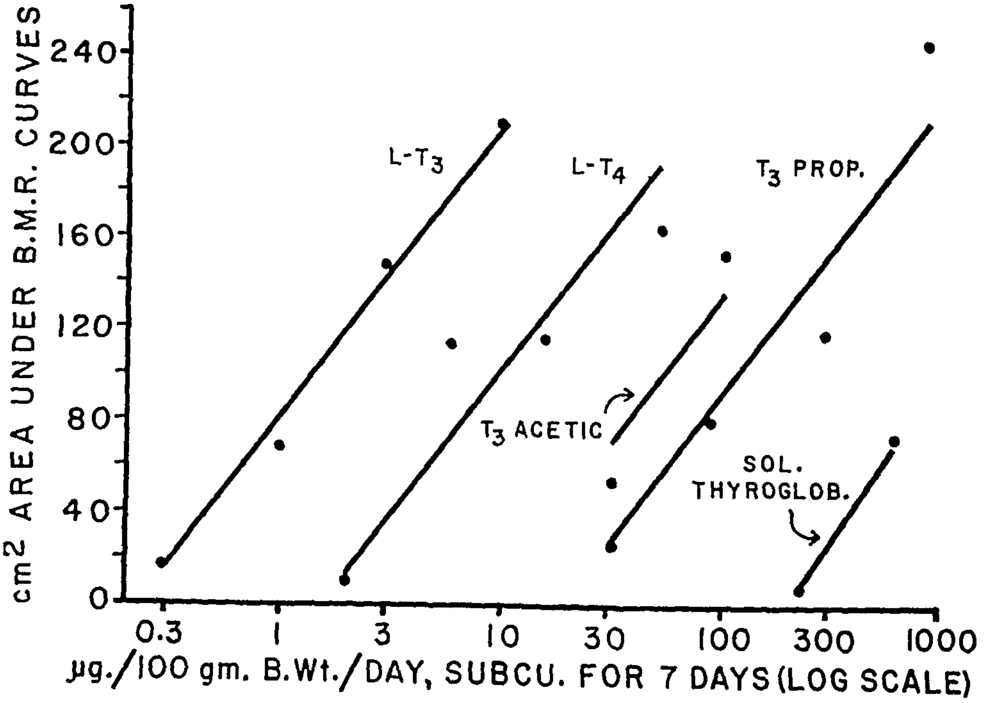
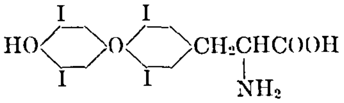
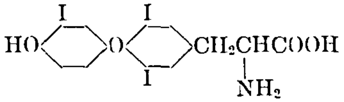
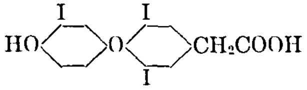
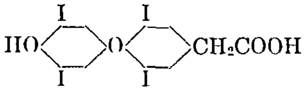
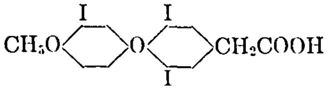
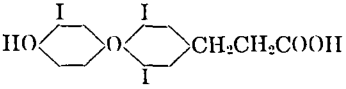
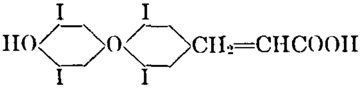
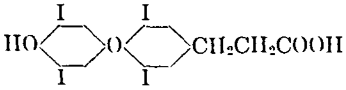
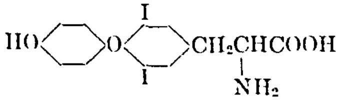
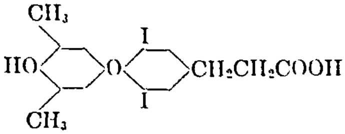
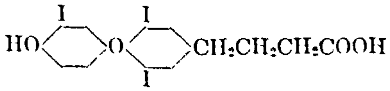
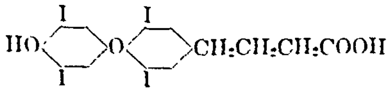


FIG. 2. *Analysis of areas under BMR curves for thyroxine analogues and soluble hog thyroglobulin:* Each curve in Figure 1 was plotted on linear graph paper such that 1 inch on the ordinate scale equaled 10 percentage points of BMR increase and 1 inch on the abscissa was equal to 1 day. The total area under each curve was determined with a polar plainmeter from Day 0 through the Day 8, where applicable. (Similar area analyses for Figure 1C and 1E were made but not shown.) Two or more points, obtained in this manner for each preparation, were plotted against the respective dose levels. Calculations for the best fitting straight lines (Y = area versus X = log. dose) were made as explained in text. A common slope value was used for each line.

the various preparations affected the oxygen consumption of rats in much the same manner. The major differences seen were among the doses of individual drugs required to produce a given level of response. These differences were more accurately assessed by determining the area-under-curve at all dose levels of each compound for a given number of treatment days. The areas and the logarithms of their corresponding doses were used to compute the best fitting straight lines (with common slope) shown in Fig. 2.

The potency of each unknown, relative to thyroxine, is listed in Table 1 where the results by goiter-prevention test are also entered for comparison of the assay methods. It will be noted that the potency values are presented in the order of decreasing activity by goiter-prevention test for all active analogues other than thyroxine. It is apparent that the calorigenic activity values are in general agreement with the goiter-prevention activity values, as to order. The activity of 3,5,3′-triiodo-*l*-thyronine was, as expected, greater than thyroxine and all the analogues. The tri- and tetraiodothyroacetic acids were antigoitrogenically equal to each other and

TABLE 1. ACTIVE THYROXINE ANALOGUES

			Relative activities	
			Goiter Prevention	Calorigenic
W1563		L-thyroxine (sodium pentahydrate)	100	100
W1575		3,5,3′-triiodo-l-thyronine	800	800
W1499		3,5,3′-triiodothyroacetic acid	51	21
W1483		3,5,3′,5′-tetraiodothyroacetic acid	57	11
W1702		3,5,3′-triiodothyroacetic acid, methyl ether	32	11
W1525		3,5,3′-triiodothyropropionic acid	28	10
W1745		3,5,3′,5′-tetraiodothyroacrylic acid	20	13
W1524		3,5,3′,5′-tetraiodothyropropionic acid	14	7
No. 37		3,5-diiodo-l-thyronine	>7	11*
W1659		3,5-diiodo-3′,5′,-dimethylthyropropionic acid	8	5
W1621		3,5,3′-triiodothyrobutyric acid	7	5
W1620		3,5,3′,5′-tetraiodothyrobutyric acid	7	5

* Value reported based on results with single dose level.

about half as active as thyroxine. Pitt-Rivers (14) also found these two analogues equally potent by goiter-prevention assay. As may be seen, at the bottom of Table 1 the tri- and tetraiodothyrobutyric acids were also equally potent. These results suggest that introduction of the fourth iodine at the 5′ position does not necessarily result in a reduction of activity in homologous series with deaminated side chains. Calorigenically, 3,5,3′-triiodothyroacetic acid appears to be more potent than its tetraiodo homologue. The difference is of borderline significance statistically. However, Barker (15) observed a similar difference in calorigenic activity for the tri- and tetraiodothyroacetic acids. The importance of the phenolic hydroxyl group in the 4′ position is reflected in the significant reduction of goiter-prevention activity of compound W1702 compared to compound W1499. The drop in activity resulted from replacement of the 4′ hydroxyl group, in 3,5,3′-triiodothyroacetic acid, with a methoxy group. The effects of side chain length on activity are also shown in Table 1. Activity decreased uniformally as the deaminated side chain length was increased from two to four carbons. Thus, the "propionic," "acrylic" and "butyric" acid analogues were each less active than the "acetic" acid analogues. This finding appears to conflict with a postulate of Selenkow *et al.* (3) which suggests decreasing activity with decreasing side chain length. It is true, however, that the "benzoic acid" analogues do not possess appreciable activity by these assay methods (Table 2) (16, 17).

The importance of iodine and its structural position with relation to activity can be seen by the assay results listed in Tables 1 and 2. It will be seen that in the thyronine series, 3-monoiodo and 3,3′-diiodo-*dl*-thyronine were inactive (Table 2) but 3-5-diiodo-*l*-thyronine had significant activity (Table 1). This latter result agrees with Selenkow *et al.* (3). In the "acetic acid" and "propionic acid" series the 3-monoiodo, 3,3′-diiodo and 3,5-diiodo preparations were inactive (Table 2). Similarly, 3,5-diiodothyrobutyric acid was found to be inactive. Therefore, it appears that, in the thyronine series, iodines in the 3,5 positions are required for significant biological activity. For preparations with deaminated side chains, iodines in positions 3,5,3′ or 3,5,3′,5′ are needed for biological activity. However, an exception to this latter observation may be seen in Table 1 where 3,5-diiodo-3′,5′-dimethyl-thyropropionic acid is shown to have significant activity. Thus, the inactive 3-5-diiodothyropropionic acid (Table 2) can be rendered active by introduction of methyl groups at the 3′ and 5′ positions (W1659, Table 1).

Active analogues—relation of antigoitrogenic to calorigenic activities

An interesting feature of Table 1 is the greater goiter-prevention potencies of several analogues compared to their respective calorigenic activities. Ratios of goiter-prevention/calorigenic assay values of these analogues are: tetraiodothyroacrylic, 1.5; tetraiodothyropropionic, 2.0; triiodothyro-

TABLE 2. ANALOGUES ASSAYING LESS THAN 3% OF LEVO THYROXINE BY GOITER-PREVENTION AND CALORIGENIC TESTS

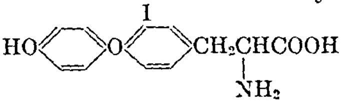
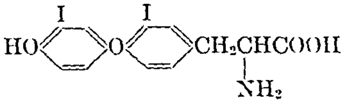
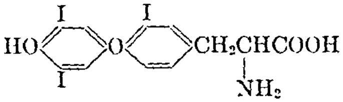
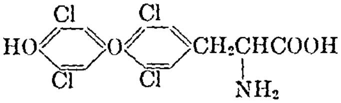
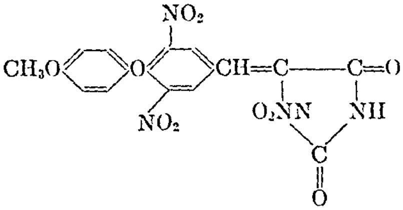
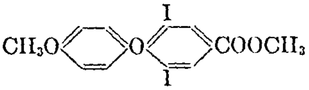
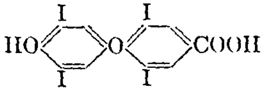
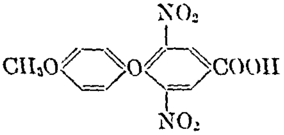
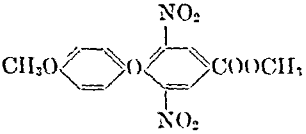
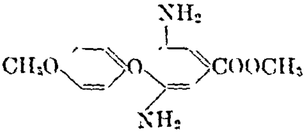
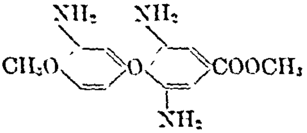
	Thyronine series	
W1631		3-iodo-dl thyronine
W1641		3,3′-diiodo-dl-thyronine
W1646		3,3′,5′-triiodo-dl-thyronine
W1633		3,5,3′,5′-tetrachloro-dl-thyronine
No. 12		1-nitro-5-(3,5-dinitrothyromethylene) hydantoin, methyl ether
	Benzoic acid series	
No. 6		3,5-diiodothyroformic acid, methyl ether, methyl ester
W1489		3,5,3′,5′-tetraiodothyroformic acid
No. 4		3,5-dinitrothyroformic acid, methyl ether
No. 5		3,5-dinitrothyroformic acid, methyl ether, methyl ester
No. 9		3,5-diaminothyroformic acid, methyl ether, methyl ester
No. 11		3,5,3′-triaminothyroformic acid, methyl ether, methyl ester

TABLE 2. (*Continued*)

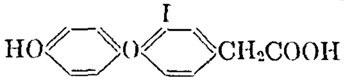
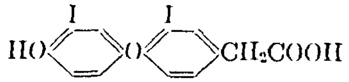
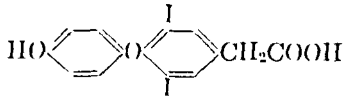
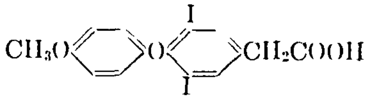
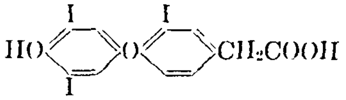
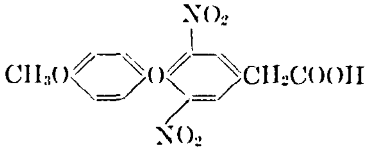
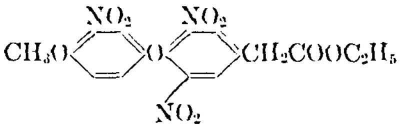
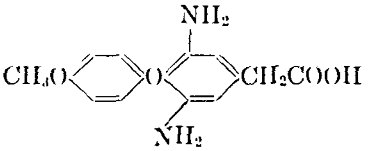
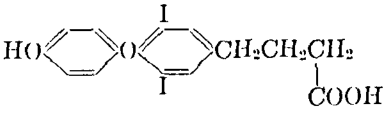
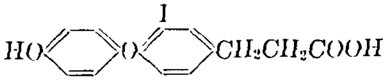
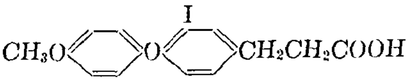
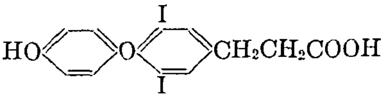
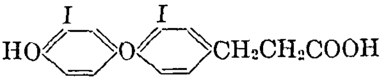
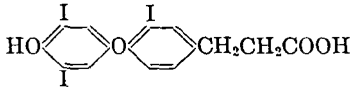
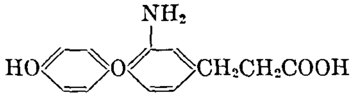
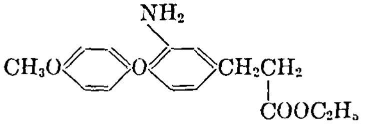
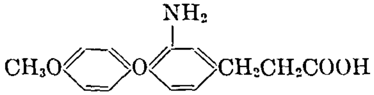
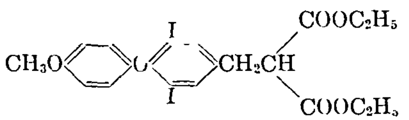
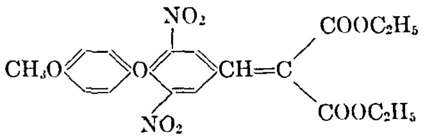
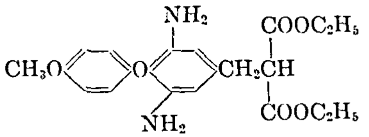
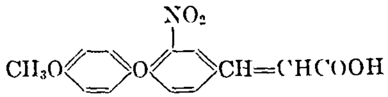
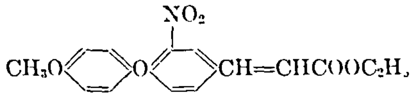
	Acetic acid series	
W1691		3-iodothyroacetic acid
W1701		3,3′-diiodothyroacetic acid
W1502		3,5-diiodothyroacetic acid
W1680		3,5-diiodothyroacetic acid, methyl ether
W1699		3,3′,5′-triiodothyroacetic acid
No. 10		3,5-dinitrothyroacetic acid, methyl ether
No. 2		3,5,3′-trinitrothyroacetic acid, methyl ether, ethyl ester
No. 15		3,5-diaminothyroacetic acid, methyl ether
	Butyric acid series	
W1618		3,5-diiodothyrobutyric acid
	Propionic acid series	
W1608		3-iodothyropropionic acid
No. 22		3-iodothyropropionic acid, methyl ether
W1607		3,5-diiodothyropropionic acid
W1622		3,3′-diiodothyropropionic acid

TABLE 2. (*Continued*)

W1628		3,3′,5′-triiodothyropropionic acid
No. 20		3-aminothyropropionic acid
W1704		3-aminothyropropionic acid, methyl ether, ethyl ester
No. 51		3-aminothyropropionic acid, methyl ether
No. 17		3,5-diiodothyromethylmalonic acid, methyl ether, diethyl ester
No. 8		3,5-dinitrothyromethylenemalonic acid, methyl ether, diethyl ester
No. 16		3,5-diaminothyromethylmalonic acid, methyl ether, diethyl ether
No. 21		3-nitrothyroacrylic acid, methyl ether
No. 24		3-nitrothyroacrylic acid, methyl ether, ethyl ester

acetic, 2.4; triiodothyropropionic, 2.8; methyl ether of triiodothyroacetic, 2.9; and tetraiodothyroacetic, 5.0. With due regard for the error of estimate in each assay method, it can be stated that ratios of 2.5 or greater may be considered statistically significant at $p = 0.95$. It is interesting that these same preparations were the ones found to be most active by the tadpole metamorphosis assay, by Money *et al.* (6, 7). Such high ratios may represent a real dichotomy of actions, for example with respect to suppression of pituitary TSH output versus stimulation of oxygen consumption, within a given species (rats), as well as between species (rats versus tadpoles).

Another possible cause of high ratios may relate to the differential calorigenic activities of several analogues in normal versus thiouracilized rats. This latter point will be the subject of a subsequent paper (18).

Active analogues—decomposition associated with method of solution

The results of repeated assays with 3,5,3′,5′-tetraiodothyroacetic acid are shown in Table 3. This close examination was made to verify the previously mentioned high goiter-prevention/calorigenic activity ratio of 5.0 and also to account for a peculiar behavior of this analogue under certain conditions of solution. In the first four assays (Table 3) the compound was dissolved in alkali and diluted with physiological saline. The resulting potencies of 56% and 69% by goiter-prevention test were observed with groups of five to eight rats each, at several dose levels. Calorigenically, the same composite lot assayed 6% in one group of four rats at a single dose level, and 11% with groups of eight rats at three dose levels. The high ratio between activities by the different assay methods was thus firmly established. After these results were obtained the method of solution was modified by using alcohol as a primary solvent, followed by alklali and physiological saline as a diluent. Many of the inactive analogues (Table 2) under test at that time were more easily solubilized by this procedure. Solutions of a new lot of tetraiodothyroacetic acid (lot 6, assays 56 and 56A, Table 3) were prepared for tests using these modifications. The compound was par-

Table 3. Effects of method of solution on biologic activity of 3,5,3′,5′-tetraiodothyroacetic acid. (W1483, T_4 acetic)

Assay No.	Lot No.	Per cent of thyrox. activity		Method of solution
		Goiter-prevention	Calorigenic	
36A	Composite (2, 3, 4)	56	—	Alkaline-saline[1]
46A	Composite (2, 3, 4)	—	6*	Alkaline-saline[1]
43	Composite (2, 3, 4)	69	—	Alkaline-saline[1]
49A	Composite (2, 3, 4)	—	11	Alkaline-saline[1]
56; 56A	6	—	<1†	Alcohol-alkaline-sal.[2]
51	2	50	—	Alcohol-alkaline-sal.[2]
51	3	54	—	Alcohol-alkaline-sal.[2]
51	4	52	—	Alcohol-alkaline-sal.[2]
51	6	47	—	*Alcohol-alkaline-sal.*[2]
62A	6	—	11	Alcohol-prop. glycol.[3]
53	6	58	—	Alcohol-prop. glycol.[3]
53	6	69	—	Alcohol-alkaline-sal.
53	6	<1†	—	Alcohol-alkaline-sal.
Average (omitting* and †):		57	11	
Range of mean (P=0.95):		50–64		

* Preliminary test: four rats: single dosage level.

† Solutions developed color reaction.

[1] Preps. dissolved in minimal alkali (N/10 NaOH) and diluted with physiological saline.

[2] Preps. dissolved in absolute ethyl alcohol (plus minimal volume of N/10 NaOH, if needed) and diluted with saline adjusted to pH 8–9. Alcohol not greater than 10%, by volume, in final solution.

[3] Preps. dissolved in absolute ethyl alcohol and diluted with propylene glycol, such that final solution was 10% and 90%, respectively, by volume.

tially dissolved by alcohol. The addition of a small volume of N/10 NaOH gave a clear amber-colored solution. When saline adjusted to pH 8–9 was added as diluent, the color of the resulting solution immediately changed to dark green. Within two minutes the color gradually returned to a clear yellow. During the course of five days of administration of this solution in a group of eight rats, no significant elevation of BMR was obtained although, in the concentration employed, the drug was expected to produce a strong response. Thus, the activity, if any, was recorded as less than 1%, as shown in Table 3.

The possibility that lot 6 might be chemically different from the composite of lots 2, 3 and 4 (Table 3, first four assays) was considered in spite of the fact that paper partition chromatography indicated that the various lots were quite similar. A new solution of lot 6, in a concentration three times greater than previously employed, was prepared using the alcohol-alkali-saline system as before. The same color changes were observed, and assay confirmed the essential lack of calorigenic activity (Table 3, No. 56A). Lots 2, 3, 4 and 6 were then assayed separately for goiter-prevention activity. Solutions, at the much lower concentrations needed in this test method, were prepared with the alcohol-alkali-saline system. No color changes were observed and, as shown by assay No. 51, Table 3, the four lots had the expected activities. The differences in activities between lots were therefore not significant, thus corroborating observations made by means of chromatographic analysis.

At this point, it was apparent that we were dealing with a preparation for which a valid assay of biological activity depended on a set of specific conditions including the drug/alkali ratio. In order to test this hypothesis, lot 6 was again assayed, by both methods, under three different conditions of solution. For calorigenic assay No. 62A, Table 3, 675.0 mg. of lot 6 were dissolved in 15 cc. absolute ethyl alcohol and diluted to 150 cc. with propylene glycol. Since no alkali was present no color change was observed. Doses of 50 μg., 150 μg. and 450 μg./0.25 cc./100 gm. body weight were administered subcutaneously daily, for seven days, using eight rats per dose level. When the areas under the resulting BMR increment curves were used to construct a dose-response curve and this was compared to the curve for the thyroxine standard, the calculated relative activity of lot 6 was 11%. This confirmed earlier assays (Nos. 46A and 49A, Table 3) of alkaline solutions of identical drug concentrations, which did not give color reactions. For goiter-prevention assay No. 53, Table 3, lot 6 was prepared in three ways. Twelve milligrams were dissolved in 50 cc. of alcohol and a 25 cc. aliquot thereof was diluted to 250 cc. with propylene glycol to complete the first solution. A second solution was prepared by addition of 2.5 cc. N/10 NaOH to the remaining 25 cc. of the alcoholic solution followed by dilution to 250 cc. with saline which had been previously adjusted to pH 8–9. No color change was anticipated and none was observed.

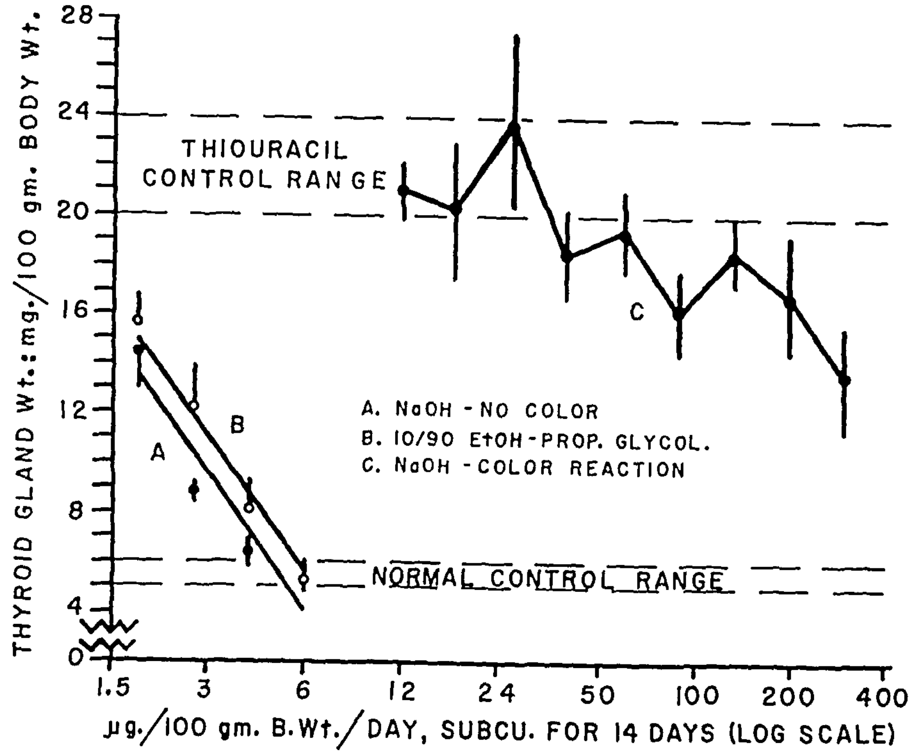


FIG. 3. *Effect of method of solution on goiter-prevention activity of 3,5,3′,5′-tetraiodothyroacetic acid:*

A. Alkaline-saline solution, pH above 11 (no color reaction).

B. Alcohol-propylene glycol solution (no alkali, no color reaction).

C. Alkaline-saline solution, pH substantially below 11 (color reaction developed).

The same lot, No. 6, was used to prepare each solution. See text for explanation of concentrations and color reactions.

The third solution, in which a color reaction was deliberately allowed to develop, was prepared by dissolving 180 mg. of lot 6 in 4.5 cc. alcohol and 3.6 cc. N/10 NaOH. Upon addition of 18 cc. of saline (pH 8–9) the expected color changes from amber to dark green and back to yellow were observed. No further change was obtained with addition of more alkali (3.3 cc.) and saline (15.6 cc.). Each of the three solutions was then assayed for goiter-prevention activity, at four or more dose levels, in groups of five to eight rats per dose level. The observed relative potencies are shown in Table 3, assay No. 53 and the dose-response curves were plotted in Figure 3. Curves A and B in Figure 3, representing solutions with and without alkali, respectively (each without color reaction) were not significantly different statistically. The relative potency estimates were 69% for the alkaline solution and 58% for the alcohol-propylene glycol solution as shown in Table 3. These results were in excellent agreement with all previous goiter-prevention assays of the various lots of tetraiodothyroacetic acid. On the other hand, Curve C, Figure 3, clearly demonstrates the expected loss of activity in a solution which gave the color changes. Bioassay calculations showed that more than 99% of the activity of this latter solution had been destroyed. This finding was substantiated by the fact that none of the original tetraiodothyroacetic acid in this solution could be detected by paper partition chromatography. Whether the minute residual activity (<1%) was due to traces of intact tetraiodothyroacetic acid, which might escape chromatographic analysis, or to biologically active decomposition products remains to be determined.

A more detailed study of the decomposition of tetraiodothyroacetic acid revealed that the speed of degradation depended on a number of variables. Among these were drug concentration, temperature, pH and trace impurities in the various lots of material (Table 3). Decomposition was effectively inhibited by the addition of hydroquinone or thyroxine and/or by lowering the temperature. It was not inhibited when an atmosphere of nitrogen and a solvent free of dissolved oxygen were used. Solutions made up at pH 11–12 and quickly frozen did not show significant decomposition. However, when the initial pH was approximately 9–10, a rapid degradation ensued, at room temperature, and the characteristic color change was obtained. Variations in pH produced concomitant variations in the speed of decomposition. Thus it was possible to obtain slow but significant destruction *without* noticeable color changes. Paper partition chromatographic analysis of such solutions revealed that the original drug had been replaced by several decomposition products including ionic iodide. Observations with other analogues indicated that N-acetylthyroxine and 3,5,3′,5′-tetraiodothyropropionic acid were similarly labile. On the other hand, triiodothyronine, thyroxine, triiodothyroacetic acid and triiodothyropropionic acid were stable under these conditions. A search of the older literature revealed that in the course of work on the structure of thyroxine, Kendall and Osterberg (19) also noted evidence of decomposition. They found that N-acetylthyroxine, unlike thyroxine itself, was unstable as its sodium salt either in solution or in the solid state.

A review of the materials and methods section of many published reports revealed that bioassays of parenterally-administered thyroxine analogues, in animals and humans, have usually been conducted with the use of alkaline solutions (2, 3 and this report). In at least one instance (5) 3,5,3′,5′-tetraiodothyroacetic acid, in alkaline solution, was autoclaved prior to injection. In view of the labile nature of certain analogues, under these conditions, it is suggested that the reported biological actions, or lack of action, attributed to these analogues may be in error. Paper partition chromatographic analysis of test solutions at the beginning and end of injection periods would appear to be a valuable adjunct to the bioassay procedures. For preparations such as 3,5,3′,5′-tetraiodothyroacetic acid, the use of solutions containing no alkali would increase confidence in the results of bioassays.

Evaluation of the decomposition problem

Evidence for the decomposition of one lot of tetraiodothyroacetic acid, in the presence of alkali, appeared late in the sequence of experiments reported here. Since most of the compounds had been dissolved in this manner, it became necessary to determine whether the other analogues, active as well as inactive, had undergone degradation. Accordingly, samples of all of the iodine-containing compounds were again dissolved in alcohol-alkaline saline and studied chromatographically[3] before and after four or five days storage in the frozen state. The pH of the freshly prepared solutions ranged from 7.8–9.8.

As expected, tetraiodothyroacetic acid (W1483, lot 6) and tetraiodothyropropionic acid (W1524) showed marked decomposition. In addition, tetraiodothyroacrylic acid (W1745), tetraiodothyrobutyric acid (W1620) and 3,5,3′-triiodothyrobutyric acid (W1621), each biologically active as shown in Table 1, may have undergone some changes. Similarly, the biologically inactive 3,5-diiodothyroacetic acid, methyl ether (W1680, Table 2) showed some evidence of decomposition. The remaining 22 analogues appeared to be stable in this chromatographic study both initially and after storage.

Since no decomposition had been observed in alcohol/propylene glycol solution, it was of interest to determine the activities of labile compounds in the absence of alkali. Comparative assays of W1483 (Table 3, Fig. 3) indicated that this compound was not more active in alcohol/propylene glycol than it was in alkaline solution when decomposition was effectively inhibited with excess alkali. Similarly, inactive W1680 (Table 2) was again found inactive when dissolved in alcohol/propylene glycol. Higher relative potencies for W1524, W1745, W1620 and W1621 (Table 1) might result if these analogues were assayed in the absence of alkali. If the degree of decomposition for a given compound is not greater than 15%, the observed potencies (Table 1), would not change appreciably because the inherent error of the goiter-prevention assay is ±15% at the 95% confidence limits.

The *in vitro* studies of Freinkel, Ingbar and Dowling (37) indicated that adsorption of thyroxine on glass surfaces might have influenced the outcome of their work with thyroxine-binding protein and thyroxine uptake by tissue. They used physiological levels of thyroxine (about 0.05 μg. to 0.1 μg./ml.) so that even slight adsorption might represent a significant proportion of the total thyroxine present. Previously, the same authors (38) had calculated that 40% of a 200 μg.% saline solution of thyroxine was adsorbed to glassware and that the percentage adsorbed decreased with increasing concentrations.

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[3] Circular paper partition chromatography, using Whatman No. 1 paper, was carried out on the samples. The solvent employed was t-amyl alcohol saturated with 6N ammonia.

Adsorption varies with different compounds. The lowest concentrations of compounds used in our experiments were generally greater than 200 μg.%. Bearing in mind the ±15% accuracy of the goiter-prevention test, *adsorption of some of the compounds may have occurred, particularly at* lower concentrations. Lack of significant adsorption may be postulated, since solutions of the same compound (thyroxine, Table 4) at different concentrations gave essentially the same results. However, preferred procedure would attempt to control the uncertainties and variables of adsorption by equilibrating glassware with preliminary solutions of all compounds.

Active analogues—speed of calorigenesis

The speed of reaction of thyroxine analogues on oxygen consumption, *in vivo* and *in vitro*, has been the subject of conflicting reports. In humans, Blackburn *et al.* (20) found that *l*-triiodothyronine produced a peak response of BMR in 24 to 48 hours versus 7 to 10 days for *l*-thyroxine, while Rall *et al.* (21) observed no qualitative differences in metabolic changes induced by thyroxine, triiodothyronine and tri- and tetraiodothyroacetic acid. The latter two analogues were reported to cause an immediate rise in oxygen consumption, *in vitro*, by Thibault and Pitt-Rivers (22). However, Barker and Lewis (23) observed no immediate metabolic increments for these two preparations either *in vitro* or *in vivo*. Parts of the curves in Figure 1 were used to construct Figure 4 in order to show, more clearly, the comparative BMR responses obtained twenty-four hours after the initial dose of several analogues and a soluble hog thyroglobulin preparation. It is apparent that the responses were roughly proportional to the dose and that each material was capable of producing significant BMR elevation as early as twenty-four hours after injection (Fig. 4, solid bars). Returning to Figure 1, it will be noted that after the final injection (indicated by arrow)

TABLE 4. COMPARISON OF "ENDPOINT" DOSAGE IN TWO BIOASSAY PROCEDURES

		Goiter-prevention[1]		Calorigenic[1]	
		No. of assays	Dose[2]	No. of assays	Dose[3]
W1563	L-thyroxine sodium pentahydrate	12	3.3	3	2.3
W1575	3,5,3′-triiodo-l-thyronine	2	0.5	2	0.25
W1499	3,5,3′-triiodothyroacetic acid	2	7.5	2	10.7
W1483	3,5,3′,5′-tetraiodothyroacetic acid	8	5.6	3	21.4
W1525	3,5,3′-triiodothyropropionic acid	3	7.7	1	19.0
W1524	3,5,3′,5′-tetraiodothyropropionic acid	1	22.5	1	37.5

[1] Dose-response curves calculated with 5–12 rats/dosage level and minimum of two dosage levels per prep.

[2] X-intercept: minimum μg. prep./100 gm. body wt./day required to maintain "normal" (6.0 mg./100 gm. B. wt.) thyroid gland weight in 200 gm. Female rats maintained on diet containing 0.3% thiouracil.

[3] X-intercept: maximum μg. prep./100 gm. B. wt./day which will not increase the oxygen consumption of 300 gm. normal male rats.

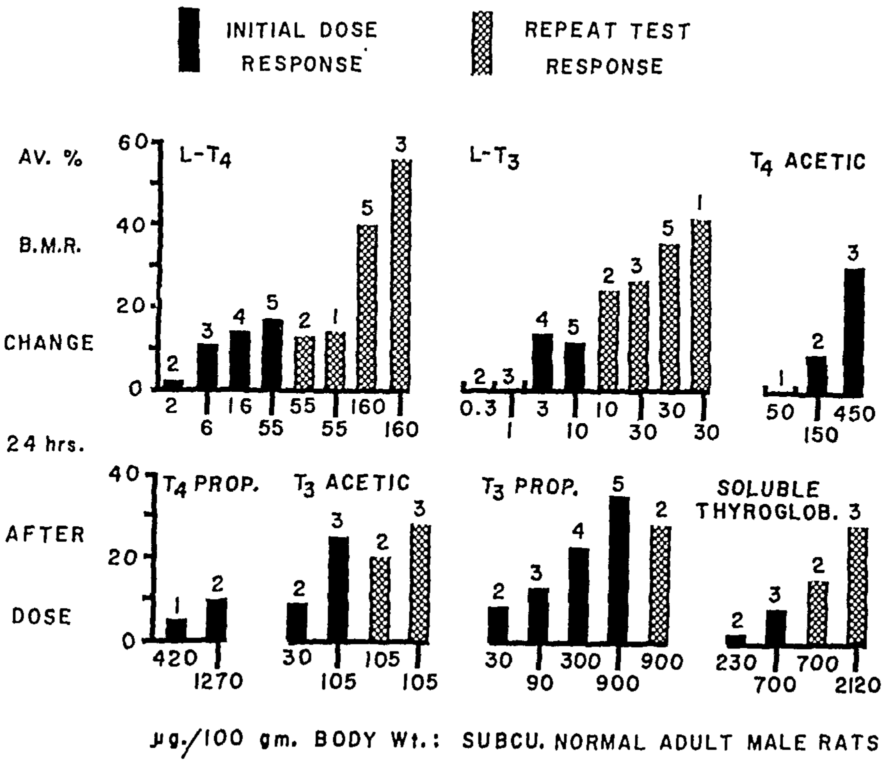

Fig. 4. *Rise in BMR, from normal base line, 24 hrs. after injection of thyroxine analogues and soluble thyroglobulin:*

Solid bars indicate responses on Day 1 shown in Figures 1 and 2.

Symbols at upper left of each series of bars identify compounds for reference to Figures 1 and 2.

Numbers over bars identify specific curves in Figure 1.

Numbers under bars identify the doses.

Cross hatched bars show the 24 hr. responses obtained in repeat tests after various groups in Figure 1 were allowed to return to their normal levels.

the rate of return to pre-treatment base lines was rapid and similar for all preparations. When the rats returned to or near their normal base line they were re-injected with doses equal to or greater than those employed originally and the twenty-four hour response was observed again as shown by the cross-hatched bars in Figure 4. These results indicate that comparable doses produced responses equivalent to those observed initially and that increased dosage resulted in greater responses for each preparation. Therefore, on the basis of BMR increments, determined twenty-four hours after dosing, no qualitative differences in response were observed with any of the materials in rats. However, it is possible that observations made earlier than twenty-four hours (e.g., 6 to 12 hours after injection) might uncover such differences since significant BMR increases have been noted in intact rats (24) as early as twelve hours after injection of soluble thyroglobulin.

The potency of soluble thyroglobulin (Figs. 2 and 4) was only 0.8% of that of thyroxine when the doses shown were used for calculation. However, this natural hormone contained less than 1% total thyroidal iodine and the biologically active thyronine-bound iodine, as determined by the method of Blau (25), represented less than one-third of the total iodine. This observation, previously reported by Kroc *et al.* (26), supported earlier results obtained by Roche and Michel (27) with thyroid glands of several mammalian species. The thyroxine standard used in this study contained about 55% total iodine and virtually all of this was biologically active. Thus, when the potency was calculated on the basis of thyronine-bound iodine contents, the activity of soluble hog thyroglobulin was 225% of that of thyroxine. Calculations indicate that a minimum of 20 to 25% of the thyronine-bound iodine in the thyroglobulin would have to be present in the form of 3,5,3′-triiodothyronine in order to account for the significant excess of biological activity.

Active analogues—antigoitrogenic and calorigenic maintenance levels

Several years ago, Dempsey and Astwood (12) showed that the maintenance level of thyroxine in rats (or the amount of thyroxine equivalent to the endogenous daily production of normal thyroids), under various environmental conditions, could be accurately assessed by the goiter-prevention method of assay. These results were obtained by noting the point at which a particular dose-response curve intersected a horizontal projection at the level of normal control thyroid weight on the ordinate. Alternatively, the point could be calculated precisely by substituting the normal control weight for Y and solving for X in the regression equation for the dose-response curve. The results thus obtained for thyroxine have been confirmed many times during the ensuing years and the procedure has achieved universal acceptance as a way of determining the activities of thyroidally active materials. More recently the precision of a relative potency estimate by this assay method was observed to be of the order of ±15% at the 95% confidence level (13).

The technical difficulties involved in BMR measurements in rats has generally precluded the usefulness of this assay method for determining maintenance or threshold levels of thyroidal preparations. Such difficulties may account for the wide difference (about 100-fold) in the thresholds of thyroxine-response for the pituitary-thyroid axis versus oxygen consumption, observed by Kennedy and Griesbach (28). Under optimal test conditions the calorigenic assay can yield values, in terms of absolute units, which agree with similar values derived antigoitrogenically. This has been demonstrated by Dempsey and Astwood (12) and Reineke *et al.* (29). Several of the goiter-prevention and calorigenic X-intercept values (bioassay "endpoints") obtained in our studies are shown in Table 4. It will be seen that results obtained by the two assay methods are in good agreement for compounds W1563, W1575, W1499 and W1524 when due allowance is made for a ±15% goiter-prevention assay error and a −33% to +50% error for the calorigenic assay. It is interesting that the latter assay gave lower results for thyroxine and triiodothyronine. The differences observed for preparations W1483 and W1525 (Table 4) were significant and appear to represent a real dichotomy of action with respect to the two parameters of function (pituitary-thyroid axis versus total metabolism). These results agree with those described in a previous section of this report.

Inactive analogues—antigoitrogenic and calorigenic tests

Thirty-three of the forty-five thyroxine analogues covered by this report were observed to have less than 3% of thyroxine-like activity, if any, as shown in Table 2. On scanning this list it will be noted that all 3- monoiodo and 3,5-diiodo compounds, which have no substituents in the 3′ and/or 5′ positions, were inactive. 3,5-diiodo-*l*-thyronine (Table 1) was the only exception to this rule. Substitution with nitro or amino groups at positions 3; 3,5; or 3,5,3′ gave inactive compounds. Similarly, 3,5,3′,5′-tetrachloro-*dl*-thyronine was found inactive. Several analogues (Table 2) having a 4′-methoxy group instead of a 4′-hydroxyl group were also tested and all were without detectable activity. This was anticipated in view of the significant reduction in goiter-prevention activity obtained with the 4′-methyl ether corresponding to 3,5,3′-triiodothyroacetic acid (Table 1).

Of particular interest in Table 2 was the fact that all 3,3′-diiodo and 3,3′,5′-triiodo analogues were singularly devoid of biological activity by either test. This observation was of special importance with regard to preparations W1641 (3,3′-diiodo-*dl*-thyronine) and W1646 (3,3′,5′-triiodo-*dl*-thyronine) since Roche, *et al.* (30) found the levo-isomers of these compounds to be 85% and 10%, respectively, as active as *l*-thyroxine by goiter-prevention test. In another paper Roche and Michel (31) reported 85% and 5%, respectively, for the *dl*-isomers of 3,3′-diiodo and 3,3′,5′-triiodothyronine versus *dl*-thyroxine by the same method of assay. Pitt-Rivers (32) found no antigoitrogenic activity with a sample of our 3,3′-diiodo-*dl*-thyronine. Similarly, Gemmill (33) observed no oral activity, calorigenically, with his own 3,3′-preparation. Recently, however, Michel (34) found that a sample of our 3,3′-preparation was of equal activity to his own 3,3′-compound by tadpole metamorphosis test, and in his goiter-prevention assay he found 30% to 40% activity, versus *dl*-thyroxine, for our material.

Michel (34) pointed out the possibility that findings of "no" biological activity for 3,3′-diiodothyronine could be due to the unusually rapid action, degradation and excretion of this analogue. Indeed, Stanbury and Morris (35) have recently demonstrated extremely high degradation and excretion rates for this preparation in man. Originally, our observations with this material were based on dosage schedules which involved one subcutaneous injection per rat per day. In view of the possibility that the lack of activity observed was due to rapid excretion and/or lability of the compound in alkaline solution, a new goiter-prevention assay was run. The 3,3′-diiodo-*dl*-thyronine was dissolved in alcohol/propylene glycol, 10/90, V/V and injected subcutaneously, three times per day per rat, at 4-hour intervals, for fourteen consecutive days. Groups of 5 to 8 rats were employed at each of six dose levels. The total dose per rat per day ranged from 30 μg. to 300 μg. At the completion of this assay it was noted that 3,3′-diiodo-*dl*-thyronine was inactive. Even the highest dose (300 μg./rat /day, equally divided into three injections per day) was unable to produce any sign of reduction in the thiouracil-induced goiters.

The discrepancy between the observations of Roche *et al.* (30), Roche and Michel (31), and Michel (34) versus those of Pitt-Rivers (32), Gemmill (33) and this report for 3,3′-diiodo-*dl*-thyronine (Table 2) suggests a possible inherent difference in assay methods which allows some investigators to find activity (30, 31, 34) while others find none (32, 33 and this report). If this were true one would expect to find similar discrepancies, between the various investigators, for other analogues. However, this does not appear to be the case. A more likely explanation for the discrepancy involves the possibility that the 3,3′-diiodo-thyronine used by Roche *et al.* (30, 31) was contaminated with 3,5,3′-triiodothyronine. Their method of synthesis for the 3,3′-diiodo compound (30) involved the deiodination of 3,5-diiodothyronine to yield 3-monoiodothyronine which was in turn iodinated to yield 3,3′-diiodothyronine. It appears conceivable that some nondeiodinated 3,5-diiodothyronine was present, as a contaminant, during the iodination of the 3-monoiodothyronine, so that not only the desired 3,3′-diiodothyronine but also some 3,5,3′-triiodothyronine would be produced. Since *l*-triiodothyronine is about eight times as active as *l*-thyroxine (Table 1) it could account for virtually all of the activity reported for 3,3′-diiodo-*dl*-thyronine by Roche *et al.* (30) assuming it were present as a contaminant to the extent of 10% by weight as the *l*-isomer. The method of synthesis employed in this laboratory by Meltzer and Stanaback (36), for the preparation of 3,3′-diiodothyronine, precludes the possibility of contamination with 3,5,3′-triiodothyronine. This was accomplished in an intermediate step, by replacing the amino group in α-benzamido-3-amino-4-(4′-methoxyphenoxy)-cinnamic acid with iodine to yield a 3-monoiodo compound free of any 3,5-diiodo contaminant. Direct iodination of the 3-monoiodo compound cannot then introduce an iodine substituent in the 5 position.

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BEHAVIOR OF IODIDE IN ACID SOLUTIONS OF HEMOGLOBIN[1]

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ABSTRACT

Acidification of aqueous solutions of oxyhemoglobin containing I^{131}-labelled inorganic iodide resulted in prompt binding of iodine to the protein, so that an appreciable fraction and quantity no longer behaved as inorganic iodide, as judged by solubility in trichloroacetic acid, dialysis and adsorption by anion-exchange resins. Binding was greatly decreased in the presence of reducing agents (thiosulfate, thiourea, ferrocyanide, ascorbic acid) and of certain halides (thiocyanate and to a lesser degree bromide), but the addition of these substances after binding had occurred failed to release iodide. Most of the bound iodine was released after brief exposure to strong alkali at room temperature. Methemoglobin, reduced hemoglobin, carboxyhemoglobin and hematin were much less effective in the binding reaction than oxyhemoglobin. Bound iodine was found in both the globin and the hemin fractions separated by the acid-acetone method. Consideration of iodine/hemin and iodine/iron ratios led to the conclusion that the iodine in the globin fraction could not be accounted for as ferriheme iodide retained in the globin. The use of trichloroacetic acid as a protein precipitant in solutions containing inorganic I^{131} could result in spuriously high values for PBI^{131} if oxyhemoglobin were present.

Although the nature of the iodine protein combination is not clear, the findings suggest that the binding is a consequence of an oxidative change resulting from the acidification of oxyhemoglobin.

IN USING the trichloroacetic acid procedure for the determination of conversion ratios (PBI^{131}/total I^{131}) in a series of arterial blood serum samples obtained one to three hours after the administration of I^{131} to human subjects, erratic results were occasionally encountered, the values in some samples being appreciably higher than had been expected and than were found in most of the samples in the series. It was noted that the high values were obtained in sera in which hemolysis had occurred, and it was found that if a crystal of sodium thiosulfate was added to such hemolyzed samples before precipitation with trichloroacetic acid, the conversion ratios were comparable to those of non-hemolyzed samples in the series. It appeared likely that the presence of hemoglobin was responsible for these curious effects and a study was made of some of the properties of solutions containing both hemoglobin and iodide.

METHODS

Hemoglobin solutions were prepared from human red blood cells obtained from outdated bank blood. After removal of the plasma by centrifugation, the cells were washed repeatedly with 0.9% sodium chloride solution and were hemolyzed by being shaken with 1 volume of water and 0.4 volumes of toluene (1). The clear hemoglobin layer was filtered through cotton, the filtrate was dialyzed against several changes of distilled water for several days at 4° C, and the resulting hemoglobin solutions were used for the experiments. Solutions containing approximately 50% methemoglobin were prepared by permitting oxyhemoglobin solutions at pH 5.1 (HCl) to undergo auto-oxidation for 24 hours at room temperature (2). Methemoglobin solutions were also prepared by adding a slight excess of potassium ferricyanide to hemoglobin solutions and then dialyzing exhaustively in tap water and then distilled water. Hemin was prepared from aqueous hemoglobin solutions by the method of Drabkin and Austin (3), and recrystallized from pyridine-chloroform (4).

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[1] Presented at the 1958 Meeting of the American Goiter Association.

The acid-acetone procedure of Anson and Mirsky (5) was used to separate hematin from globin. Oxyhemoglobin solutions made .05 to 0.1N in HCl were passed through an anion-exchange resin column and 10 to 15 volumes of acetone containing 1% 1N HCl were added to the effluent to precipitate the globin and dissolve the hemin. Precipitation was carried out in large centrifuge tubes, the supernatant solution was removed by aspiration, and the precipitate washed twice with acetone-HCl and twice with acetone before being dissolved in H_2O to appropriate volume and used for further study. Hemin was precipitated from the chilled acetone supernatant solutions by addition of 1/100 volume of 2N sodium acetate, the precipitate being collected by centrifugation and washed several times with dilute acetic acid. Recovery of hemin was not quantitative.

Radioactivity measurements were made with a well-type scintillation counter, appropriate corrections being applied for variation in sample volume. The radioactive iodine used was carrier-free Na I^{131} in isotonic saline, containing benzylalcohol (0.9%), obtained as sterile solution Sodium Radio-Iodide (I^{131}) Therapeutic from Abbott Laboratories. This material was greatly diluted with distilled water before it was used. In experiments involving trichloroacetic acid (TCA) precipitation of proteins and determination of radioactivity in the precipitate, the dissolved precipitate was counted in the test tube in which the original sample had been measured and in which precipitation and washing had been subsequently performed, so that no transfer of material was required. Precipitation of proteins was made by adding 4 to 8 volumes of ice-cold 10% TCA; ten minutes later the mixture was centrifuged, the supernatant liquid removed by aspiration, the precipitate resuspended in 10% TCA, centrifuged, and the liquid again aspirated. The precipitate was then dissolved in 2N NaOH with mild heating.

In some experiments the anion-exchange resin Amberlite IRA-400 was used to remove inorganic iodide from hemeprotein solutions. A column of resin 60×8 mm. was used, the previously recycled resin being washed with water after having been converted to the acetate or chloride form. One or 2 ml. of solution were added to the column and the effluent (10 to 12 ml.) collected in a graduated centrifuge tube, four or five portions of distilled water being used to wash the column after the sample had run in. This procedure was adequate to recover in the effluent all the heme-colored material which had been placed on the column. Passage of dilute labelled iodide solutions through the column resulted in retention by the column of more than 99% of the I^{131}.

Hemoglobin was determined by the spectrophotometric cyanmethemoglobin method (6) and in some cases by iron determination. Methemoglobin was determined by the method of Heilmeyer (7) and of Evelyn and Malloy (8). Hemin in acetone-HCl solution was estimated spectrophotometrically from the extinction coefficients of Lewis (9). Iron was determined by the method of Lorber (10) as modified by Paul (11).

Paper electrophoresis was carried out between horizontal glass plates on Whatman 3 MM paper (12). Paper chromatograms were made by the ascending method, butanol-acetic acid-water and butanol-ammonia-ethanol being used as solvents (13). Radioautographs were prepared from paper electrophoretograms and chromatograms as previously described (14).

TABLE 1. TCA-PRECIPITABLE I^{131} IN HEMOGLOBIN-IODIDE SOLUTION

Solutions (1.1 ml.) containing hemoglobin (1.44 μM/ml.), carrier-free Na I^{131} and the substances shown were treated with TCA, and the fraction of I^{131} precipitated determined.

Test substance (M/L)	Per cent of I^{131} TCA precipitate
Control	30.6
Perchlorate (.01)	30.6
Thiocyanate (.01)	6.0
Cyanide (.02)	24.3
Thiosulfate (.003)	4.2
Sulfate (.1)	32.0
Ferrocyanide (.001)	7.5

RESULTS

When carrier-free Na I^{131} was added to hemoglobin solutions and the proteins precipitated by TCA, an appreciable portion of the I^{131} present was found in the protein precipitate. The presence of reducing agents (thiosulfate, thiourea, ferrocyanide) and of thiocyanate added to the system prior to precipitation greatly decreased the proportion of I^{131} bound to protein, while perchlorate and cyanide had little effect (Table 1). Binding of I^{131} to the proteins seemed to depend upon acid conditions since passage of neutral hemoglobin-iodide solutions through anion exchange columns yielded virtually no I^{131} in the effluent while prior acidification (0.1N acetic or hydrochloric acid) of the iodide-containing hemoglobin solution resulted in emergence of a considerable quantity of I^{131} from the column with the proteins. The importance of acid in the binding was also shown in the experiments summarized in Table 2. Five ml. aliquots of hemoglobin containing carrier-free Na I^{131} (0.18 μc.) were placed in cellophane dialysis bags which were then immersed in 50 ml. buffer at pH 6.8 or in 0.1N acetic acid; the systems were kept at 4° C with occasional shaking for 72 hours, and at this time the bag contents/medium concentration ratio of I^{131} was 1.49 in the neutral medium, while the corresponding value at acid pH was 8.4. The values for serum and for saline were 1.96 and 1.04 respectively. The increased volume of the bag contents in experiment 2 (column III) suggested that an increase in the number of non-diffusible molecules had occurred, perhaps attributable in part to the cleavage of the hematin from the protein. Since 60% of the I^{131} within the bag was TCA-precipitable in the case of hemoglobin at acid pH, while only 4% of the I^{131} in the serum

TABLE 2. EQUILIBRIUM DIALYSIS OF HEMOGLOBIN-IODIDE MIXTURES

I	II	III Bag		IV Medium		V	VI Bag I^{131}	
Bag (5 ml.)	Medium (50 ml.)	Volume (ml.)	I^{131} (c. m./ml.)	Volume (ml.)	I^{131} (c./m./ml.)	Bag medium concentration ratio I^{131}	% of total	% TCA precipitable
1 HbO_2 (4% in water, I^{131})	.05M P buffer, pH 6.8	8	4535	47	3035	1.49	20.2	25
2 HbO_2 (4%, in water, I^{131})	.10N acetic acid	20	7475	35	889	8.4	82.9	60
3 Normal serum (1:1 in water, I^{131})	.05M P buffer, pH 6.8	7	5785	48	2945	1.96	22.3	4
4 0.9% saline (I^{131})	.05M P buffer, pH 6.8	5	3240	50	3130	1.04	9.4	0

bag was precipitable, it seemed that the concentration gradient in the acid-hemoglobin system was not one predominantly involving iodide.

Since these results suggested that the TCA-precipitability of iodide in hemoglobin solutions depended upon the acidic character of the protein precipitant rather than upon a hemoglobin-iodide interaction prior to precipitation, the remainder of the study was concerned with the characterization of the heme-protein iodide interaction at acid pH.

A. *Iodide concentration.* The effect of varying the concentration of iodide upon the TCA precipitability of the iodine was studied in acidified mixtures of hemoglobin and iodide. In order to make the conditions of exposure to acid solutions more reproducible, the solutions were made up to be .05N in HCl before TCA precipitation. Figure 1 shows the results of such an experiment, the hemoglobin concentration being maintained constant.

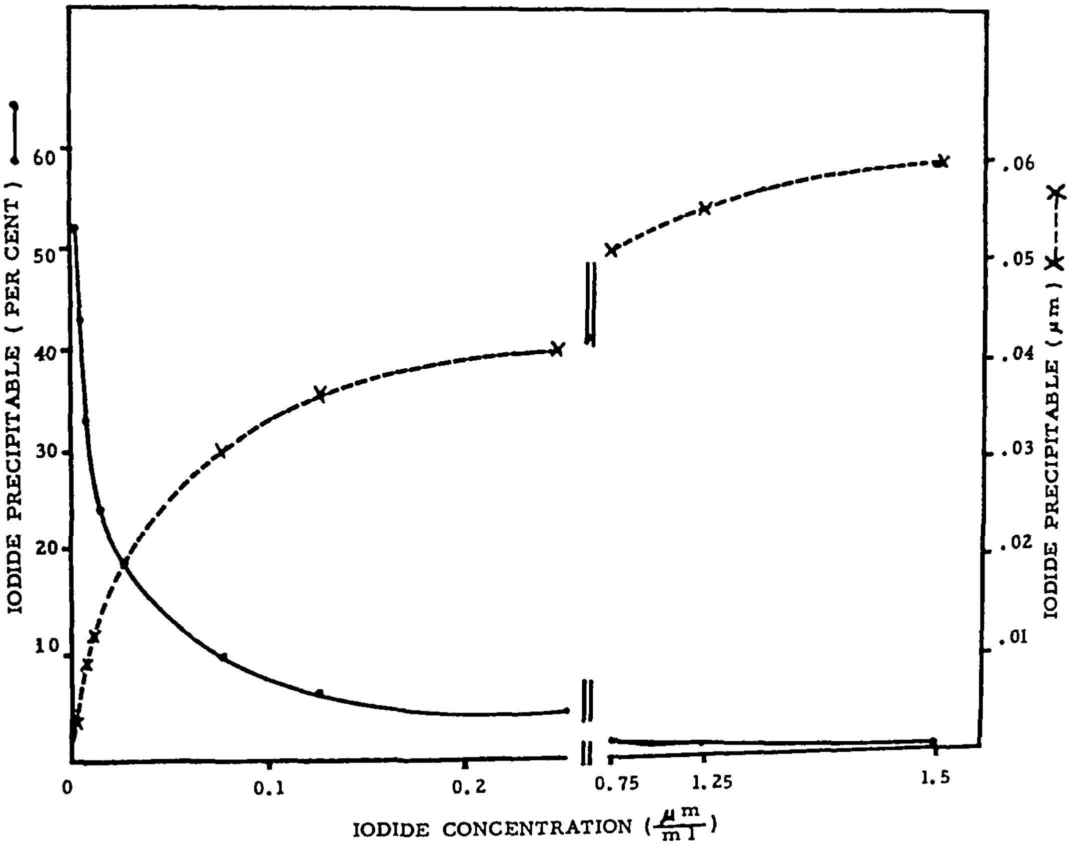


Fig. 1. *Effect of iodide concentration upon iodine precipitability.*

Potassium iodide in amounts ranging from 0 to 10 μM were added to 1 ml. portions of aqueous solutions containing 4.0 μM hemoglobin and 0.15 μc. I^{131}, the final volume being 2 ml.; 2 ml. 0. 1N HCl was then added, and after 10 minutes at room temperature the proteins were precipitated by 10% TCA and the precipitable I^{131} determined. The concentration of iodide was calculated from the amount present in 4 ml. volume.

The unbroken line represents the percentage of the I^{131} present which was precipitated with protein; the broken line indicates the quantity of iodine precipitated (calculated from the known specific activity of iodide in the system). Note change in abscissa scale.

The proportion of the I^{131} which became protein-bound varied from 53% in the absence of added carrier to less than 1% at the highest iodide concentration. However, despite the progressive reduction in the proportion of iodine bound, the quantity of $iodide^{127}$ bound increased progressively as the concentration of stable iodide rose; thus, at iodide concentration of 0.0025 μM/ml. 0.0044 μM iodine was protein-bound, while at iodide concentration 2.5 μM/ml. 0.06 μM was bound.

B. *Hemoglobin concentration.* The effect of varying the concentration of hemoglobin upon the TCA precipitability of iodide in the solution under conditions of constant iodide concentration was studied, and the results appear in Figure 2, which indicates the proportion of the iodide precipitable as the concentration of hemoglobin ranged between 0.007 and 1.4 μM/ml., at two concentrations of iodide (B and C) and also in the absence of added carrier iodide (A). The general shape of these curves was similar to that of Figure 1. At each level of iodide concentration the proportion of the total iodide which was bound increased with increasing hemoglobin concentration. Although the fraction of the iodide bound increased progressively as the concentration of hemoglobin rose, the efficiency of the process tended to decrease since the highest values for the molar ratios of iodine bound/hemoglobin present were observed at the lowest concentrations of hemoglobin. Thus, (curve B, iodide concentration .001 μM/ml.,) at hemoglobin concentration .007 μM/ml. the molar ratio of iodine bound /hemoglobin present was .0031 while at hemoglobin concentration 0.7 μM/ml., this ratio was .00052. As was also seen in Figure 1, at a given hemoglobin concentration a greater fraction of the iodide was precipitable in the more dilute iodide colutions, and the relative positions of curves B′ and C′ confirmed the finding that higher iodide concentration yielded larger quantities of bound iodine. For example, a ten-fold increase of iodide concentration increased the value for the number of moles of iodine bound per mole hemoglobin present at .007 μM hemoglobin/ml. from .0031 to .0129.

C. *Presence of other anions.* The nature and quantity of certain anions in the hemoglobin-iodide solution may modify the proportion of iodide which is precipitable. This was suggested by the observation that if hemoglobin-I^{131} solutions were treated with equivalent concentrations of different acids prior to precipitation with TCA, the percentage of I^{131} precipitable was not the same in each case. For example, pre-treatment of identical aliquots of solutions containing hemoglobin and labelled iodide with 0.1N acetic, hydrochloric, sulfuric and phosphoric acids resulted in the precipitation of 54, 30, 42 and 42% of the I^{131}, respectively, while these values were 42 and 26%, respectively, when 1N acetic and 1N hydrochloric acid were used.Pre-treatment of hemoglobin solutions with sodium chloride-hydrochloric acid mixtures of the same pH but varying ionic strength also suggested an inhibitory effect of the chloride ion.

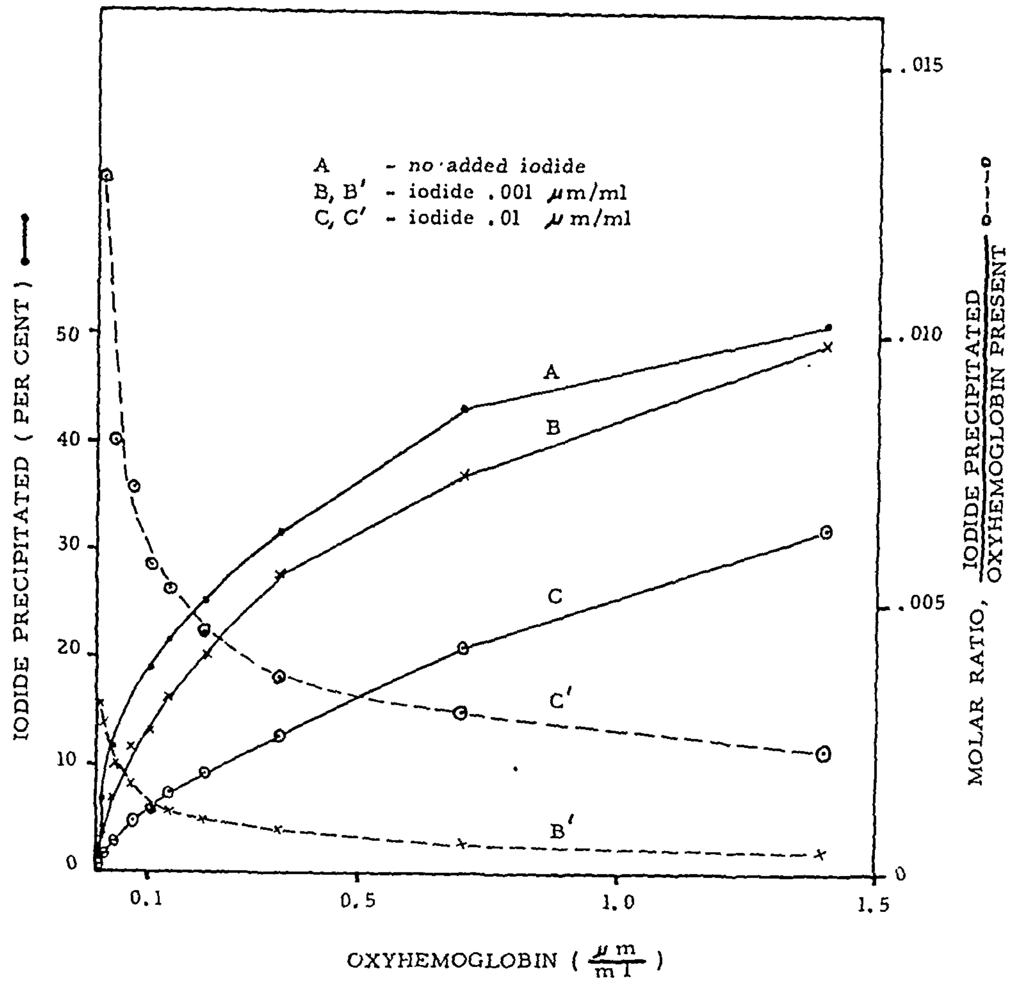


FIG. 2. *Effect of hemoglobin concentration upon iodine precipitability.*

Increasing amounts of hemoglobin ranging from 0.035 to 7.5 µM were added to test tubes containing the same quantity of labelled iodide. Three parallel experiments are shown; tubes of series A contained no added carrier iodide, tubes of series B contained .005, and of series C .05 µM KI^{127} respectively. The total volume of solution was 2.5 ml. To each tube 2.5 ml. 0.1N HCl was then added; one hour later the proteins were precipitated by 10% TCA and precipitable I^{131} determined. The concentrations of iodide and of hemoglobin are calculated from the quantities present in 5 ml. volume.

The percentage of the total I^{131} bound to protein is shown in the unbroken lines (left ordinate), and the values for the moles of iodine precipitable per mole of oxyhemoglobin present are shown in the broken lines (right ordinate), the values in B′ and C′ being calculated from the known specific activities in the corresponding series B and C.

The comparative effects of the presence of chloride, bromide, fluoride, thiocyanate and iodide on the binding of iodine are shown in Figure 3. These ions were added as sodium salts to solutions of hemoglobin and labelled iodide which were then made .05N in HCl and the proteins precipitated with TCA. Under the conditions of the experiment fluoride and chloride had little effect while the presence of bromide caused a fall in the proportion of I^{131} precipitable with protein; at a bromide concentration of

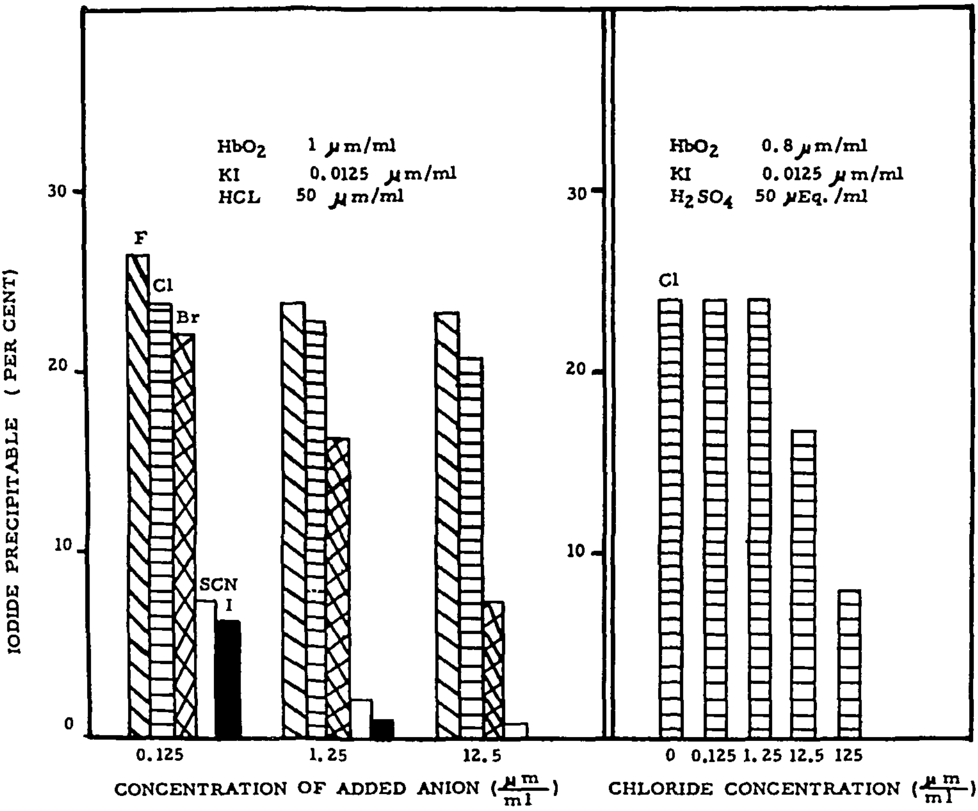


FIG. 3. *Effect of added halides on precipitability of* I^{131}.

Left panel. To a series of tubes containing 4.0 μM HbO_2 and 0.05 μM KI labelled with I^{131} were added fluoride, bromide, chloride, thiocyanate, and iodide, in amounts of 0.5, 5.0, and 50.0 μM as sodium salts, the final volume being 2.0 ml. 2.0 ml. 0.1N HCl was then added to each tube, and after 30 minutes at room temperature, TCA precipitation was performed. The percentage of the total I^{131} precipitated is shown.

Right panel. Experiment similar to A, but the concentration of HbO_2 was smaller, 0.1N H_2SO_4 was used in place of 0.1N HCl, and the concentration of chloride was varied from 0 to 125 μM/ml.

12.5 μM/ml. only one-third as much iodide was precipitable as in the absence of bromide. The inhibitory effect of added chloride (which had probably been masked by the high chloride concentration resulting from the use of hydrochloric acid as the acidifying agent) is apparent in the right panel of Figure 3, where sulfuric acid was used in place of hydrochloric, and a chloride concentration of 12.5 μM/ml. appreciably reduced iodine binding. The great inhibitory effect of thiocyanate upon I^{131} precipitability is shown in Figure 3; the binding of iodine was reduced to 30, 8 and 2% of control values by the three concentrations of thiocyanate tested. The inhibitory effect of thiocyanate is comparable in magnitude to that of iodide itself on the protein-binding of I^{131}, although the iodide ion appeared somewhat more effective. Comparison of the effects of bromide and iodide indicated that iodide contamination of bromide did not account for the inhibitory effects of the latter; since 12.5 μM/ml. of bromide yielded a value for precipitable I^{131} similar to that encountered at 0.125 μM/ml. of added iodide, 1% iodide contamination of bromide would have been required to account for the effects on this basis.

The inhibitory effect of thiocyanate depended not only upon its concentration but also upon the concentration of hemoglobin. This was shown in a study of the effect of thiocyanate upon the TCA precipitability of I^{131} in two hemoglobin-Na I^{131} (carrier-free) systems, the first containing 1.5 Mμ HbO_2/ml. and the second 0.15 μM/ml. In the more concentrated hemoglobin solution, inhibition was found to be 10, 25 and 70% at thiocyanate concentrations of 10^{-4}, 10^{-3}, and 10^{-2} M respectively, while the corresponding values in the more dilute solution were 43%, 58%, and 84%; per cent inhibition was defined as $100 \times (1 - \%I^{131}$ precipitated in presence of thiocyanate/$\%I^{131}$ precipitated in absence of thiocyanate).

D. *Effect of other heme compounds.* Comparison of iodine binding by some heme proteins and by hemin indicated that oxyhemoglobin was by far the most effective substance tested. Table 3 indicates the effects of hematin, oxyhemoglobin and methemoglobin in equimolar concentration in producing TCA insolubility of iodide, under various conditions. An appreciable fraction of the I^{131} was precipitated with hematin only in the absence of carrier iodide; the addition of globin to hemin prior to dissolving in dilute NaOH did not increase the proportion of I^{131} bound. Thiocyanate, thiourea and dithionite are seen to have exerted some inhibitory effect on the carrier-free Na I^{131}-hematin system. Methemoglobin was quite ineffective in binding iodine in acid solution; it is possible that the slightly higher percentage of I^{131} bound in the presence of dithionite than in the control may have been due to oxygenation of reduced hemoglobin. The ineffectiveness of methemoglobin was also apparent in experiments similar to those in Figure 2, in which mixtures of oxyhemoglobin and methemoglobin prepared by auto-oxidation of the former, were used at various concentrations

TABLE 3. TCA-PRECIPITABLE I^{131} IN SOLUTIONS OF HEMATIN, HbO_2, MetHb

The test substances were added to solutions of carrier-free Na I^{131} containing the designated quantities of heme compounds, HCl was added to a final volume of 2.5 ml. and concentration of .05 N, and TCA-precipitable I^{131} determined. Hematin solution was prepared just before use by dissolving recrystallized hemin in 3.1 equivalents of NaOH. MetHb solution was a well-dialyzed sample of ferricyanide-treated hemoglobin.

Substance added (μM)	Hematin, 3.45 μM	Oxyhemoglobin, 3.45 μM	Methemoglobin, 3.45 μM
	% of I^{131} TCA-precipitable		
Control	8.6	56.8	0.9
KI 0.01	1.0	40.0	0.5
KI 0.10	1.0	15.2	0.4
KI 1.00	1.0	3.1	0.4
NaSCN 1.0	2.7	7.1	0.4
Thiourea 1.0	0.7	30.6	0.5
$Na_2S_2O_4$ (excess)	0.5	0.9	1.3

and in different proportions; the curve expressing the relationship between the fraction of I^{131} bound and the HbO_2 concentration of the mixture was very similar to that found in solutions of oxyhemoglobin alone. As indicated in Table 1, ferrocyanide was found to exert a considerable inhibitory effect on the binding; it was therefore essential that solutions of methemoglobin prepared by the ferricyanide method be dialyzed very thoroughly to ensure the absence of both ferri- and ferro-cyanide from the system before using the methemoglobin in experiments on iodine binding, particularly where the effect of the methemoglobin on binding by oxyhemoglobin-containing solutions was studied.

Figure 4A compares equimolar concentrations of oxyhemoglobin, reduced hemoglobin and carboxyhemoglobin with respect to iodine precipitability, and shows the greater proportion bound by oxyhemoglobin. To demonstrate the decreased effectiveness of reduced hemoglobin in this system it was found necessary to equilibrate with nitrogen for 10 to 15 minutes and to exclude air from the system with great care.

In Figure 4B is shown the difference in results observed when acid was added to the oxyhemoglobin solution after, as compared with 10 minutes before, the addition of iodide to the system, the chloride and HCl concentration being the same in the two cases; the experiment indicated again that conversion of oxyhemoglobin to acid hematin in the presence of iodide led to considerable binding of iodine while little bound iodine resulted from the re-acidification in iodide solution of the neutralized acid hematin formed in the absence of iodide.

E. *Iodine in globin and in hemin.* In order to determine whether the bound iodine in the oxyhemoglobin-iodide-HCl system was in the porphyrin or in the globin fraction, the acid-acetone procedure was used as illustrated in Figure 5. The fractions were designated as follows: original solution of HbO_2-iodide^{131}-HCl, A; the anion-exchange column effluent, B; the washed acetone precipitate derived from acetone treatment of the column effluent, G; the acetone supernatant, F; and the hemin derived from the latter, H. The results of 14 experiments such as shown in Figure 5 are summarized in Table 4. These experiments were made under a variety of conditions of temperature, time and concentration of reactants. In 10 experiments in which iodide carrier was used, between 22 and 60% of the iodine in A was not adsorbed by the column (and hence was presumably not iodide) and appeared in B; the values for the TCA-precipitable I^{131} in the A solutions in general were slightly less than those for the I^{131} not retained by the column. An average value of 33% (range 29–43) of the I^{131} emerging from the column was accounted for in the globin precipitate.

The globin precipitate, G, could be redissolved in water and reprecipitated by acetone-HCl with relatively small losses of radioactivity. For example, in one experiment three equal aliquots of the column effluent, B, were subjected to the acid-acetone procedure and acetone-washed fraction

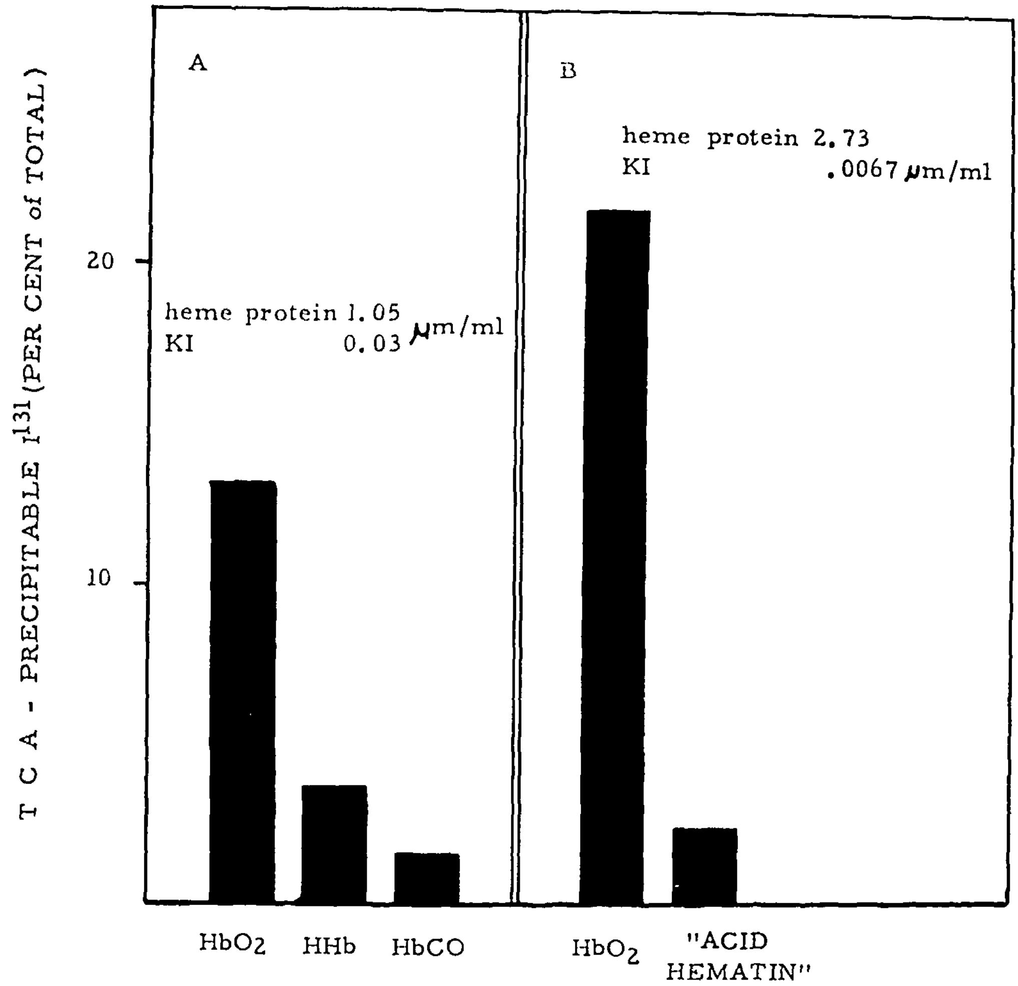


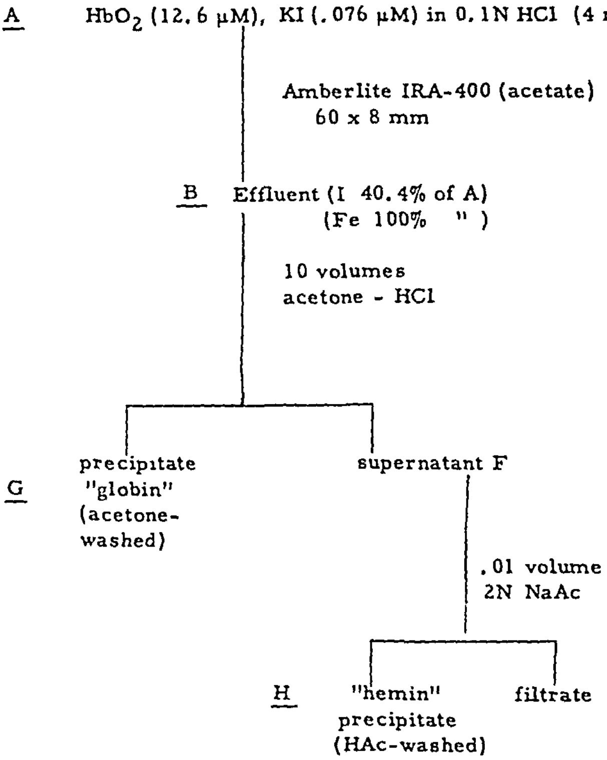
FIG. 4. *TCA precipitable I^{131} in heme protein solutions.*

A. Air, nitrogen, and carbon monoxide were bubbled into solutions containing 6.32 μM HbO_2, 0.182 μM labelled KI and 3 drops octyl alcohol in 4 ml. volume. After 10 minutes and while gas flow continued, 2 ml. 0.1 ml. 1N HCl was injected into each solution via a rubber tube and then TCA precipitation was performed.

B. "Acid hematin" was prepared by adding 0.1 ml. 1N HCl to 1 ml. HbO_2 solution (4.2 μM) and allowing it to stand 10 minutes at room temperature. After neutralization of the mixture with 0.13 ml. 1N NaOH, 0.01 μM labelled KI was added, the solution again made 0.1N in HCl and after 10 minutes, TCA precipitation was done. The HbO_2 control was treated in the same way, except that 0.1 ml. 1N NaCl was used instead of 1N HCl, and water instead of NaOH.

G prepared from each; analysis of one sample was made directly, another after dissolving the precipitate and re-precipitating, and of the third after dissolving and reprecipitating twice; the once, twice and thrice precipitated globin fractions contained 39.6, 35.6 and 32.4% of the I^{131} in the column effluent, respectively, while the corresponding iron values were 14.9, 13.6, and 13.6% of B.

In 6 experiments in which hemin was prepared from the acetone filtrate



	I (% of B)	Fe (% of B)	I/Fe (mM/M)
B	100	100	2.44
G	43.4	16.6	6.34
F	56.6	83.4	1.65
H	26.6	58	1.11

FIG. 5. Distribution of iodine and iron in acetone-soluble and insoluble fractions of acidified oxyhemoglobin.

the washed, isolated (but not recrystallized) hemin accounted for 42.2% (range 25–57) of the I^{131} in F.

The presence of reducing agents (thiourea, ascorbic acid) in solution A prior to acidification resulted in almost complete removal of I^{131} by the resin column, so that little I^{131} appeared in B, a result comparable to the diminution in the fraction of iodine precipitable by TCA under similar circumstances. In an experiment identical with experiment 4 in Table 4, except that thiourea 40 μM/ml. was present in the HbO_2-iodide solution before acidification, only 3.2% of the I^{131} in A appeared in B, and G contained only 4.4% of the RaI in B, in contrast to corresponding values of 22 and 29% in the absence of thiourea.

The "globin" (G) prepared by the procedure described had a brownish color, and its concentrated solutions were yellow; in alkaline solution and in the presence of a reducing agent (dithionite), the absorption spectrum of G was similar to that of globin hemochromogen but the maxima were shifted 4–5 mμ toward the shorter wavelengths (maxima at 554 and 523 mμ); the material exhibited peroxidase activity by the benzidine test; and when G was prepared from hemoglobin solution to which a reducing agent had been added prior to acidification, and binding of iodine had been

TABLE 4. IODINE AND IRON IN "GLOBIN" PREPARED FROM RESIN-TREATED ACID SOLUTIONS OF OXYHEMOGLOBIN AND IODIDE

A, B, and G refer to the fractions so designated in Figure 4.

Expt.	HbO_2	KI	HCl	I^{131} in column effluent	I^{131} in "globin" (G)	Fe in globin	I^{131}/Fe*
	μM/ml.	μM/ml.	M/L	(% of A)	(% of B)	(% of B)	
1	3.15	0.019	0.1	40	43	17	2.53
2	2.63	0.018	0.1	30	29	16	1.81
3	2.58	0.019	0.05	33	36	14	2.57
4	3.0	0.019	0.055	22	29	18	1.61
5	5.3	0.011	0.09	28	29	—	—
6	3.1	0.02	0.10	22	31	12	2.58
7	2.32	0.003	0.09	30	27	—	—
8	2.7	0.004	0.17	24	30	11	2.73
9	2.0	0.0044	0.05	38	41	16	2.56
10	2.2	0.005	0.06	60	35	—	—
					av.: 33	av.: 14.9	I^{131}/Fe =2.34 av.
11	2.26	no carrier	0.05	59	34	—	—
12	3.05	no carrier	0.10	60	42	—	—
13	2.9	no carrier	0.10	78	44	18	2.44
14	2.63	no carrier	0.10	76	45	19	2.36
					av.: 41.3	av.: 18.5	I^{131}/Fe =2.40 av.

$$* \; I^{131}/Fe = \frac{I^{131} \text{ in } G(\% \text{ of } B)}{Fe \text{ in } G(\% \text{ of } B)}.$$

largely prevented, the acetone precipitate was much lighter in color. Since hemin (ferriheme chloride) can be readily converted to iodohemin (i.e. ferriheme iodide) (15), and since it has been claimed (9, 16) that acidification of oxyhemoglobin and subsequent extraction with acetone or ether liberates only part of the hemin from the protein unless a reducing agent is present, the possibility was considered, in view of the aforementioned observations suggesting that the "globin" was not free of hematin, that the I^{131} in the globin fraction was actually bound to hematin not separated from the globin by the acid-acetone procedure, particularly since some I^{131} was found in the hemin isolated from the acetone soluble fraction in these experiments. This possibility was explored in two ways: (1) by determination of iron in the globin precipitate as well as in A and B, and by comparison of the iron and iodine content of these fractions, and (2) by studying the hemoglobin-iodide^{131} system at various values of pH and comparing the fraction of I^{131} and of hemin in the acetone soluble and insoluble fractions.

(1) Iron analysis indicated that usually all the iron in A was recovered in the column effluent B, while an average of 14.9% of the effluent iron was present in fraction G (Table 4). The iron analyses were in good general agreement with spectrophotometrically determined values for hemin in the acetone filtrate. The value for the fraction of I^{131} present in G was always greater than the corresponding iron value, the ratio being 2.3 (Table 4).

Since the I^{131}/Fe ratio was always higher in the "globin" than in the acetone supernatant, in the column effluent, or in the hemin (see insert, Fig. 5), it seemed unlikely that the I^{131} bound to the globin fraction could be accounted for on the basis of ferriheme iodide adherent to the acetone precipitate.

(2) Parallel observations were made of the effect of varying the pH of HbO_2-I^{131}-HCl solutions upon I^{131} binding and splitting of hemin from the protein. These experiments were modelled after those of Lewis (9) and the results are shown in Figure 6. Solutions of HbO_2 and I^{131} of constant ionic strength were maintained at pH values ranging from 6.5 to 2.0 and aliquots of each solution were used to determine the fraction of the total I^{131} bound (by the anion-exchange resin method), the fraction precipitable by TCA and by acetone, and the proportion of the total hemin soluble in acetone. As the pH decreased, a larger fraction of the I^{131} became bound (curve II) the binding becoming appreciable at pH 4.5 and continuing to rise to pH 2.0. In general the curve describing TCA-precipitability (IV) paralleled curve II. The plateau of curve IV between pH 6.5 and 4.8 could represent the acidifying effect of the precipitant itself. It was of interest to find that maintenance of the solutions at pH 4 to 2 with mineral acid resulted in progressively higher values for the TCA precipitable I^{131} possibly because acidification with TCA alone may not have permitted completion of the reaction before the protein was thrown out of solution. The curve representing I^{131} bound was similar to that of hemin split from the protein (curve V), suggesting that the formation of acid hematin and the binding of iodine occurred *pari passu*. However, below pH 3.5, 75–90% of the hemin was split from the protein while 25–30% of the total I^{131} present was in the acetone precipitate (curve I). When the I^{131} in the acetone precipitate was expressed as a fraction of the bound I^{121} (curve III), it was apparent that in the pH range 2–3 45% of the bound I^{131} was in the protein precipitate, which at most contained only 15% of the hemin. The increase in bound I^{131} despite achievement of a plateau for the percentage of acetone-precipitable I^{131} suggested that at the lower pH values ferriheme itself bound iodide, and this view was consistent with the finding that TCA-precipitable I^{131} always exceeded the acetone-precipitable I^{131}. Figure 6 also shows that the presence of a reducing agent (ascorbic acid) (points marked A) not only sharply decreased the binding of I^{131} but also permitted quantitative cleavage of hematin from the acidified oxyhemoglobin. The results suggest that the binding of I^{131} by the heme protein paralleled the formation of acid hematin, but quantitative considerations suggest, as do the I/Fe ratios in globin, that iodohemin adherent to the protein did not account for the I^{131} bound to globin.

A few observations have been made concerning some of the properties of the bound iodine in the globin precipitates and column effluents of acidified hemoglobin-iodide^{131} solutions. TCA precipitation of these solutions

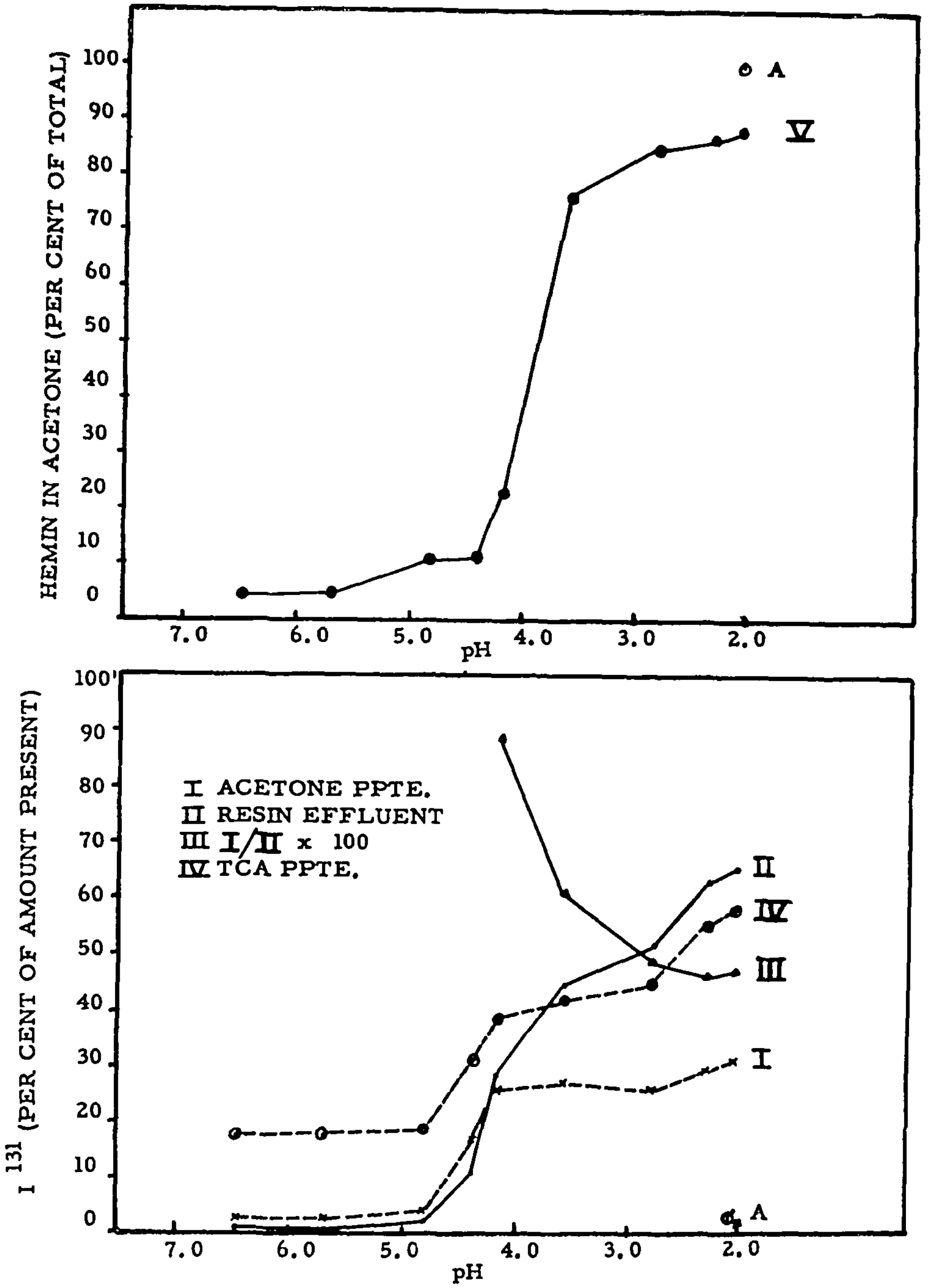


FIG. 6. *Effect of pH on I^{131} bound to protein and on separation of hemin from globin.* Solutions of oxyhemoglobin (0.77 μM/ml.) containing carrier-free NaI^{131} were adjusted to various pH, ionic strength being maintained constant (.033) by appropriate NaCl-HCl mixtures. Aliquots were used to obtain the data shown. Curve I represents the percentage of the total I^{131} precipitable by acetone at each pH, curve II the percentage not adsorbed by anion-exchange resin, curve IV the percentage precipitable by TCA. Curve III is derived from I and II and indicates the fraction of bound I^{131} present in the acetone precipitate. Curve V (upper panel) shows the percentage of the total hemin which was present in the acetone solutions.

The points marked A are values for a solution to which ascorbic acid was added prior to acidification to pH 2.06.

showed 70–80% of the I^{131} in both B and G to be precipitable and similar values were found on permitting B and G to pass through anion-exchange columns, 70–80% of the I^{131} present emerging from the column. Dialysis of solutions of the labelled acetone precipitate (within the bag) against 10 volumes of distilled water resulted in bag/medium I^{131} concentration ratios of 15 to 25. After prolonged dialysis of labelled G solutions the I^{131} within the bag was still not completely TCA precipitable. These results suggested some instability in neutral and acid solutions of the linkage by which iodine was bound to protein.

The addition of large quantities of iodide, thiocyanate or reducing substances to labelled B or G solutions did not change the proportion of I^{131} that was TCA-precipitable. A brief exposure to 0.5N NaOH, at room temperature, however, decreased the percentage of I^{131} precipitable by TCA or not retained by anion-exchange columns to 10–20% of the values prior to NaOH exposure.

Chromatographic studies of B and G after pancreatic enzyme hydrolysis failed to reveal the presence of labelled mono- or diiodotyrosine, thyroxine or triiodothyronine. Chromatograms (butanol-acetic acid-water) of unhydrolyzed B showed a benzidine-positive, brown spot near the solvent front which contained I^{131} and this was tentatively identified as hemin; most of the radioactivity and pigmented material remained at the origin, but a faint radioactive zone in the iodide region was also usually detectable. Paper electrophoresis of G at pH 5.5 for two hours revealed most of the I^{131} to be at the origin, as was the protein but some I^{131} had the mobility of iodide. Pre-treatment of G with strong alkali and partial neutralization prior to electrophoresis resulted in intensification of the iodide zone and considerable diminution of the activity at the origin. More prolonged electrophoresis at acid pH (18 hours) resulted in a long cathode-migrating smear in which protein (Durrum reagent), peroxidase activity (benzidine) and radioactivity were superimposable. In general, the chromatographic and electrophoretic results confirmed the impression gained from other experiments that the I^{131} was present in both hemin and globin, and that alkali released iodide from the bound form.

DISCUSSION

These experiments indicate that appreciable quantities of iodine may become bound—i.e. may no longer behave as inorganic iodide, as judged by TCA-precipitability, dialyzability and behavior toward anion-exchange resins—when iodide-containing solutions of oxyhemoglobin are acidified. The iodine was found in both the protein and the hemin, the proportion and amount varying with conditions of pH, concentrations of reactants, ionic strength, and other factors. Although some hemin or a hemin derivative was present in the globin fraction prepared under conditions leading to iodine binding, quantitative considerations failed to support the idea that all the protein-bound iodine was iodohemin. Using the observations that the "globin" contained, on the average, 2.3 times as much of the total bound iodine as of the iron in the acid-iodide-oxyhemoglobin system, that 15% of the total hemin was retained in the "globin," that only 80% of the I^{131} in G was TCA precipitable, and assuming that all the I^{131} in the acetone filtrate was hemin-I^{131}, one may calculate that only 45% of the globin iodine could be accounted for as iodohemin. This calculation is based on the assumption that all the heme molecules in hemoglobin are equal in their iodine-combining capacity. There is some evidence that one of the hemes in methemoglobin may be dissimilar to its fellows in its behavior toward acid cleavage (9), and it is perhaps possible that the hemin firmly bound to the globin could have a larger iodine/iron ratio than existed in the hemin that was extractable by acetone.

The effect of bromide chloride and particularly of thiocyanate in decreasing the binding of iodine could in part be related to competition between these anions and iodide for the formation of halide derivatives of hematin, but the observation that only a fraction of the bound iodine was accounted for as iodohemin suggests that this does not completely explain the inhibitory mechanism. The failure of cyanide appreciably to affect iodine binding in this system may be related to the very limited dissociation of HCN at the low pH of the reaction mixture.

The reasons for the variation among the values listed in Table 4 for the fraction of the total iodine found in the acetone precipitate is not entirely clear. The experiments differed in concentration of reactants, pH and ionic strength, which are factors known to influence the results. It is also possible that the temperature at which acidification occurred and the time elapsing between acidification and precipitation, factors which were not controlled in these experiments, could be important. The inconstancy of the iodine composition of the globin-iodine compound, as reflected in the diminishing iodine content resulting from repeated precipitations, is another factor that might be responsible for variation.

The curves expressing the relationship between the quantity of iodine bound and both the iodide concentration and the hemoglobin concentration (Figs. 1 and 2) appear to be of exponential nature. Re-plotting the data of curves B′ and C′ in Fig. 2 as a log-log graph indicated a straight line relationship between log (quantity of iodine bound) and log (hemoglobin concentration) at each iodide concentration; a similar graphical method applied to the data of Figure 1, i.e. plotting the log (quantity of iodine bound) against log (concentration of iodide) did not yield a straight line, and it is therefore likely that an equation of the Freundlich adsorption isotherm type does not describe the relationship between these quantities.

Although hemin (via alkaline hematin) could combine with trace quantities of iodide under the conditions of these experiments, of all the heme compounds tested (HbO_2, HHb, HbCO, MetHb, and hemin) oxyhemoglobin was by far the most effective in the iodine-binding system. These observations together with the failure of acid hematin to combine appreciably with iodide, suggested that (ferrous) heme attached to globin and combined with oxygen was required for maximal iodine binding consequent to acidification. It is known that certain reducing substances (ascorbic acid, epinephrine) cannot be accurately determined in hemolyzed sera deproteinized by trichloroacetic acid, and it has been suggested that the liberation of "active oxygen" which occurs when oxyhemoglobin is acidified leads to oxidation not only of the ferrous iron but also of oxidizable substances in the solution, including globin itself (17). Acidification of oxyhemoglobin fails to free globin entirely of hemin, and some of the pigment is very firmly bound to the protein, perhaps by a reactive group formed as a consequence of globin oxidation (16, 18); if a reducing agent is present or if oxyhemoglobin is converted to a non-oxygen-containing heme protein prior to acidification, the globin can be almost completely freed of heme (9, 16). In addition to this mechanism of oxidation by oxygen released from combination, it has also been suggested that hydrogen peroxide may be formed when oxyhemoglobin is acidified, and the peroxide may oxidize appropriate substances that may be present, hematin acting as peroxidase (19). The importance of the oxidative change in effecting the binding of iodine was implied not only by the effectiveness of oxyhemoglobin as compared with other heme proteins but by the effect of reducing agents in decreasing the binding. The studies of Lemberg (19) of the oxidation of ascorbic acid by solutions of oxyhemoglobin undergoing acidification emphasized the rapidity both of the reaction and of the disappearance of the oxidizing system after acidification, features which were characteristic also of the oxyhemoglobin-iodide-acid system. It is not clear from the data available whether the inhibition of iodine binding by reducing agents such as ferrocyanide or thiosulfate is attributable to the presence of an oxidizable substance which may spare iodide or to a direct reduction by these substances of an oxidized form of iodine.

The nature of the protein-bound iodine is not known. The preliminary chromatographic and electrophoretic studies have indicated the presence of labelled hemin, labelled protein, and, after hydrolysis, inorganic iodide. The alkali-lability of the binding, its greater stability in acid, the non-dialyzable character of the bound iodine and the apparent lack of effect of reducing agents upon the compound once formed are striking features. Instability in alkali is not characteristic of iodotyrosines and no evidence of these compounds was detected. If oxidation of iodide to iodine by active oxygen were the mechanism of iodination, the low pH at which the iodination occurred might conceivably prevent iodination of tyrosine residues, since alkaline conditions facilitate this reaction (20), which proceeds very slowly below pH 3 (21). An iodination that could occur at acid pH is the formation of a sulfenyl iodide, by the reaction between iodine and a thiol group (20). Although sulfenyl iodides are considered unstable, evidence has recently been presented indicating the formation and persistence of this grouping as a result of the reaction of iodine with tobacco mosaic virus (22).

The fact that even at very low iodide^{127} concentrations seldom was more than 50 to 60% of the available iodine bound suggested that the sequence might possibly be:

$$2I^- \xrightarrow{\text{oxidation}} I_2 \xrightarrow{+RH} HI + RI$$

Until characterization of the products has been achieved, such an idea is, of course, speculative.

It is believed from quantitative considerations mentioned above, that although the iodine-globin compound was not free of hemin (or a derivative), one may conclude that the iodine-protein binding may occur as a consequence of the same oxidation effects which produce adherence of hemin to the protein rather than that adherent iodohemin is necessarily the form in which iodine occurs in the protein.

Some aspects of the iodine-protein combination bear a superficial resemblance to iodine metabolism of the thyroid gland; in particular the capacity of the oxyhemoglobin solution to abstract iodine from a surrounding medium at low pH (Table 2), the considerable inhibitory effects of thiocyanate and thiourea, and the dependence upon "oxidative conditions." Despite the unphysiological pH at which this reaction occurs and other obvious differences from either thyroidal "trapping" or organic binding of iodine, one wonders whether conditions might not exist under which iron porphyrin proteins such as peroxidase or the cytochromes might bind iodine and serve as an iodide transport mechanism.

The iodine binding that can occur when oxyhemoglobin undergoes conversion to acid hematin, together with the observations on hemolyzed sera mentioned in the introductory statements, suggested that the use of TCA as a protein precipitant might yield spuriously high values for PBI^{131} in systems containing oxyhemoglobin and iodide^{131}. Incubation of homogenates of blood-containing organs, such as spleen, with I^{131} was sometimes associated with the occurrence of appreciable I^{131} in the TCA-precipitate of such a system (23). The effect of the presence of various concentrations of hemoglobin in serum containing iodide^{131} upon the conversion ratio as determined by the TCA method is seen in Figure 7. At a serum hemoglobin concentration of 500 mg.%, 10% of the I^{131} was precipitable and this was almost completely prevented by addition of thiourea prior to precipitation. Hemolysis of this degree might therefore cause an error of 10 to 40% in serum conversion ratios of euthyroid subjects. Serum seemed to exert some inhibitory effect on the TCA-precipitability of iodine in hemoglobin solutions as shown by the higher values for TCA-precipitable I^{131} in the absence of serum (Fig. 7); possible causes of this inhibition, which would

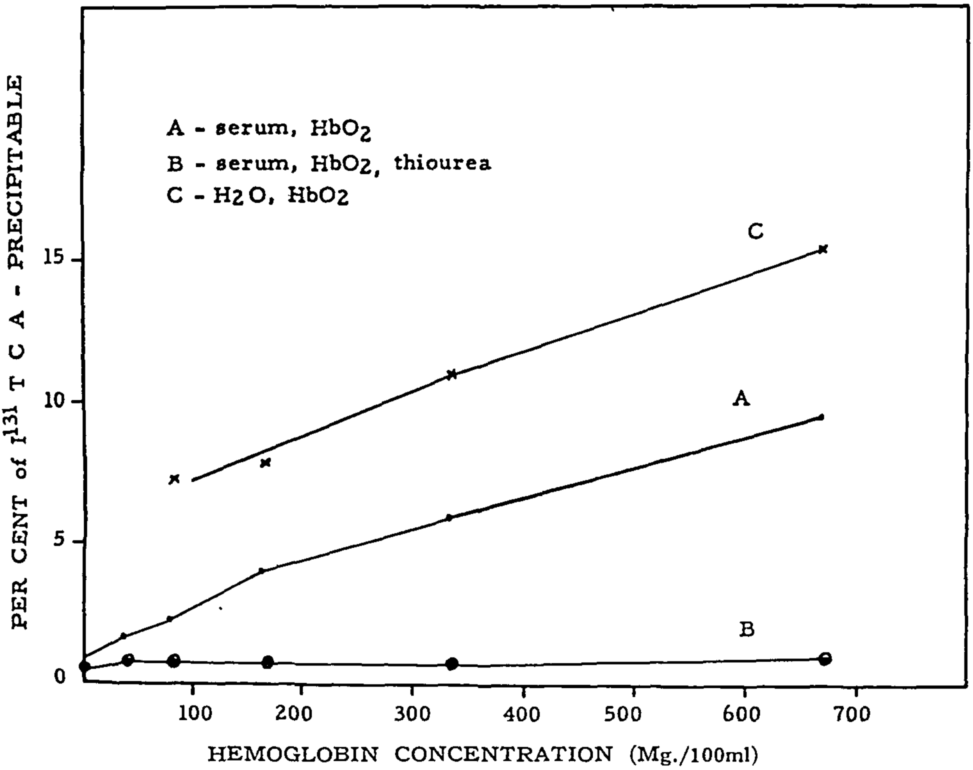


Fig. 7. *TCA precipitability of serum iodide-I^{131}; effect of added hemoglobin.* Aliquots of serum (0.5 ml.) containing carrier-free NaI^{131} were adjusted to different hemoglobin concentrations, the total volume being 1.5 ml., and proteins were precipitated by TCA (A). B is same as A save for the presence of thiourea (3.3 mgm./ml.) added prior to TCA. C represents hemoglobin and NaI^{131} in absence of serum.

decrease the precipitation error that might otherwise occur, include the presence of reducing substances, inorganic iodide and other halides.

Although analyses of TCA-precipitable I^{127} in serum containing hemoglobin were not made, one might anticipate from the data shown in Figures 1, 2 and 7 that increased values for PBI^{127} might be encountered in serum samples containing a high concentration of hemoglobin, particularly if the serum iodide^{127} concentration were also high.

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COMPARISON OF THYROIDAL AND GASTRIC IODIDE PUMPS IN RATS[1]

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ABSTRACT

Low iodine intake activated the thyroidal iodide pump of rats but had no effect on the gastric pump. Thyroid/serum (T/S) and gastric juice/serum (J/S) radioiodide concentration ratios were similar in rats receiving adequate amounts of dietary iodine. When graded doses of NaI^{127} or $NaClO_4$ were injected, the T/S was more markedly depressed at any dose level used than was the J/S. In rats on a diet with adequate iodine content the capacity of the rat's stomach juice for "pumped" iodide was found to be higher than that of the thyroid. Analysis of the findings suggests that the gastric iodide pump differs from its thyroidal counterpart a) by not being responsive to TSH and to low serum iodide levels, and b) by having a smaller affinity for iodide.

SINCE transport of iodide across the stomach wall is not followed by incorporation of the iodide into thyroglobulin, the process can be studied without recourse to the methods of "chemical dissection" used in investigating the thyroidal iodide pump. It would be convenient if the gastric and the thyroidal iodide pump were in every respect comparable, and results obtained regarding the nature, kinetics and regulation of the former could be held valid for the latter as well. Indeed, gastric and thyroidal iodide transport do show similarities: a) at physiologic serum iodide levels stomach juice/serum and thyroid/serum iodide concentration gradients of rats are approximately the same (vide infra); b) both mechanisms are inhibited by thiocyanate (unpublished observations) and perchlorate (1) in rats, as they are in other species (2, 3, 4). On the other hand thyrotrophin (TSH), to which the thyroidal iodide pump is exquisitely sensitive (5, 6, 7), has no unequivocal effect on gastric iodide transport (8). This paper points out further differences between the gastric and the thyroidal iodide pump of rats with regard to their responses to a) low iodine intake, b) elevation of serum iodide levels and c) graded doses of perchlorate.

MATERIALS AND METHODS

Young adult male Holtzman rats were used. For three weeks they were fed either a Remington diet (Low Iodine Test Diet, Nutritional Biochemicals Corporation, Cleveland, Ohio) or the same diet fortified with 1 mg. of iodine (as NaI) per kg. They were fasted for 24 hours before the experimental procedures, which were the following: a) ligation of pylorus and cardia, and either nephrectomy or, in most instances, tying off

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of all the structures in the renal hilum performed in order to obviate the influence of renal excretion on the equilibration of I^{131} (9); the operations were carried out under ether anesthesia; b) subcutaneous injection of 12 mg. of propylthiouracil (PTU) in 0.6 ml. of alkaline solution immediately after closure of the abdominal incision, and c) subcutaneous injection of a tracer dose of I^{131} one-half hour later. I^{131} was given in one ml. of isotonic (154 mM/l.) NaCl solution, or with graded doses of NaI^{127} or $NaClO_4$. Both NaI^{127} and $NaClO_4$ were prepared in isotonic solutions. When the dose given was less than the NaI^{127} or $NaClO_4$ contained in one ml. of this solution, the injected volume was made up to one ml. with isotonic NaCl. The rats which received the highest dose of NaI^{127} were injected with five ml. of the stock solution. In all cases of NaI^{127} administration the tracer dose of I^{131} was thoroughly mixed with the solution of NaI^{127} given, and specific activity was determined. Four hours after the injection of I^{131} the rats were killed. Blood was obtained from the abdominal aorta, the gastric contents were collected, and the thyroid was dissected out and weighed on a Roller-Smith balance. One-tenth ml. of serum and of the supernatant of the centrifuged gastric contents, as well as the thyroid, were prepared for radioactivity determination by the method of VanderLaan and Greer (5).

The following abbreviations will be used:

T = I^{131} (or labelled I^{127}) per 100 mg. thyroid
S = I^{131} (or labelled I^{127}) per 0.1 ml. serum
J = I^{131} (or labelled I^{127}) per 0.1 ml. gastric juice

Several pilot experiments were carried out on rats fed the fortified Remington diet or Rockland pellets for three weeks, subjected to the operative procedures listed above, and injected with a tracer dose of I^{131} one-half hour after the administration of 12 mg. of PTU. a) Since some of our rats received NaCl while others did not, the effect of this salt on gastric and thyroidal iodide transport was examined. As much as 5 ml. of isotonic NaCl given with the I^{131} had no effect on gastric or thyroidal iodide concentrating ability. Controls received 5 ml. of isotonic $Na_2S_2O_3$. The J/S in these groups was 31.8 ±2.6 (S.E.)(9 rats) and 38.9±4.0 (10 rats) respectively. b) J/S ratios 6 hours after the administration of I^{131} were no greater than at 4 hours. Numerous earlier observations have indicated that the T/S ratio becomes stable within an hour or less after the injection of I^{131}. The J/S and T/S values to be presented can therefore be regarded as equilibrium concentration ratios. c) Attempts were made to correlate the amount of gastric juice secreted in 4 hours with the J/S, since the volume of the supernatant of the centrifuged gastric juice from animals used in this study varied from only a little over 0.1 ml. to 5–6 ml. No correlation between the J/S and the amount of gastric juice secreted could be established. Because of this and the lack of systematic variation in the amount of gastric juice among groups receiving different forms of treatment, rate of gastric juice secretion was not regarded as a heteropoietic factor in this study. d) In four rats, 92±1.5 (S.E.)% of the I^{131} of the gastric juice (four hours after the injection of tracer amounts) was found in the supernatant when 1 part of centrifuged stomach juice was precipitated with 9 parts of 10% trichloroacetic acid this agrees with chromatographic findings (10) which showed 90–95% of the I^{131} in gastric juice to be in the form of iodide. In six rats 83±5.2% of serum I^{131} was not precipitated by trichloroacetic acid four hours after the injection of the tracer. This was presumably not due to the presence of hormonal I^{131} in the serum, since PTU had been given in sufficiently large amounts to prevent thyroid hormone formation. Ingbar and Freinkel (11) have also found that about 20% of I^{131} added to human serum is not dialyzable. Binding of I^{131} in serum and gastric juice was neglected in the interpretation of our results. It is unlikely that the error thus introduced exceeded 10% in any group.

For the computation of the concentration of "pumped" I^{127} in thyroid and gastric

TABLE 1. THYROID/SERUM (T/S) AND GASTRIC JUICE/SERUM (J/S) I^{131} CONCENTRATION RATIOS IN RATS FED A REMINGTON DIET OR THE SAME REGIMEN FORTIFIED WITH IODINE (1 MG./KG.)

Diet	No rats	Body wt., gm	Thyroid wt., mg	T/S	J/S
Remington	8	236 ±4*	17.2 ±1.4	151.4 ±10.1	30.3 ±6.0
Remington + I	9	247 ±7	11.5 ±0.5	31.1 ± 1.4	36.4 ±4.3

* Mean ± standard error

juice the I^{127} which had entered by diffusion was estimated as $\frac{1}{3}$ and $\frac{3}{4}$, respectively, of the serum I^{127} concentration (1), and was subtracted from the total concentration of I^{127} (Table 2, Fig. 1).

RESULTS

Table 1 shows that the T/S was greatly increased in rats on a low iodine diet, which confirms earlier observations (12, 13), but that the J/S in the same rats was no greater than in rats which had received adequate amounts of iodine.

When I^{131} was given with graded doses of I^{127} (Table 2), the T/S was more readily depressed than the J/S. Figure 1 shows that in the gastric juice, as in the thyroid, the concentration of "pumped" I^{127} rose and then tended to level off as serum I^{127} was elevated; the plateau, which indicates the capacity for "pumped" I^{127}, was more than five times as high for the gastric juice as for the thyroid. The highest concentration of "pumped" I^{127} in the gastric juice of any animal in this study was 350 µg./0.1 ml., compared with 46 µg./100 mg. in the thyroid which had taken up the largest amount of iodide. Whereas the average J/T ratio was close to 1 when only a tracer dose of I^{131} was given to rats fed the Remington diet with added iodine, this value rose to 5.5 when serum I^{127} was increased to about 20 µg./0.1 ml., and declined again when serum I^{127} levels were further elevated (Fig. 2). This decrease at very high serum I^{127} levels was a predictable finding. If iodine entered both gastric juice and thyroid by diffusion only, a J/T of about 2.3 (see broken line in Fig. 2) would be expected on the basis of studies with large doses of perchlorate (1). The J/T would tend to approach this value as serum I^{127} levels are raised so high as to flood the thyroidal and gastric pumps and eventually render diffusion of iodide into thyroid and gastric juice preponderant over active transport.

TABLE 2. EFFECT OF NaI^{127} ON THYROIDAL AND GASTRIC IODIDE PUMPS IN RATS FED A REMINGTON DIET WITH IODINE (1 MG./KG.) ADDED

No rats	Body wt., gm	Dose of NaI^{127} µM	Serum I^{127} (S) µg/0.1 ml	Thyroidal "pumped" I^{127} (I minus S/3) µg/100 mg	"Pumped" I^{127} in gastric juice (I minus 3S/4), µg/0.1 ml	T/S	J/S
9	247 ±7*	0	—	—	—	31.1 ±4.4	36.4 ±4.3
7	254 ±8	6.16	0.71 ±0.11	14.3 ±1.5	18.1 ± 3.5	21.8 ±2.6	31.5 ±7.9
6	242 ±11	30.8	3.5 ±0.33	15.9 ±2.1	56.7 ±13.4	5.5 ±1.3	16.5 ±2.6
8	240 ±9	154.0	22.4 ±1.4	23.9 ±3.3	157.1 ±34.5	1.4 ±0.35	7.7 ±1.7
5	228 ±15	770.0	132.2 ±2.1	36.5 ±5.6	178.7 ±52.0	0.62 ±0.12	2.1 ±0.4

* Mean ± standard error.

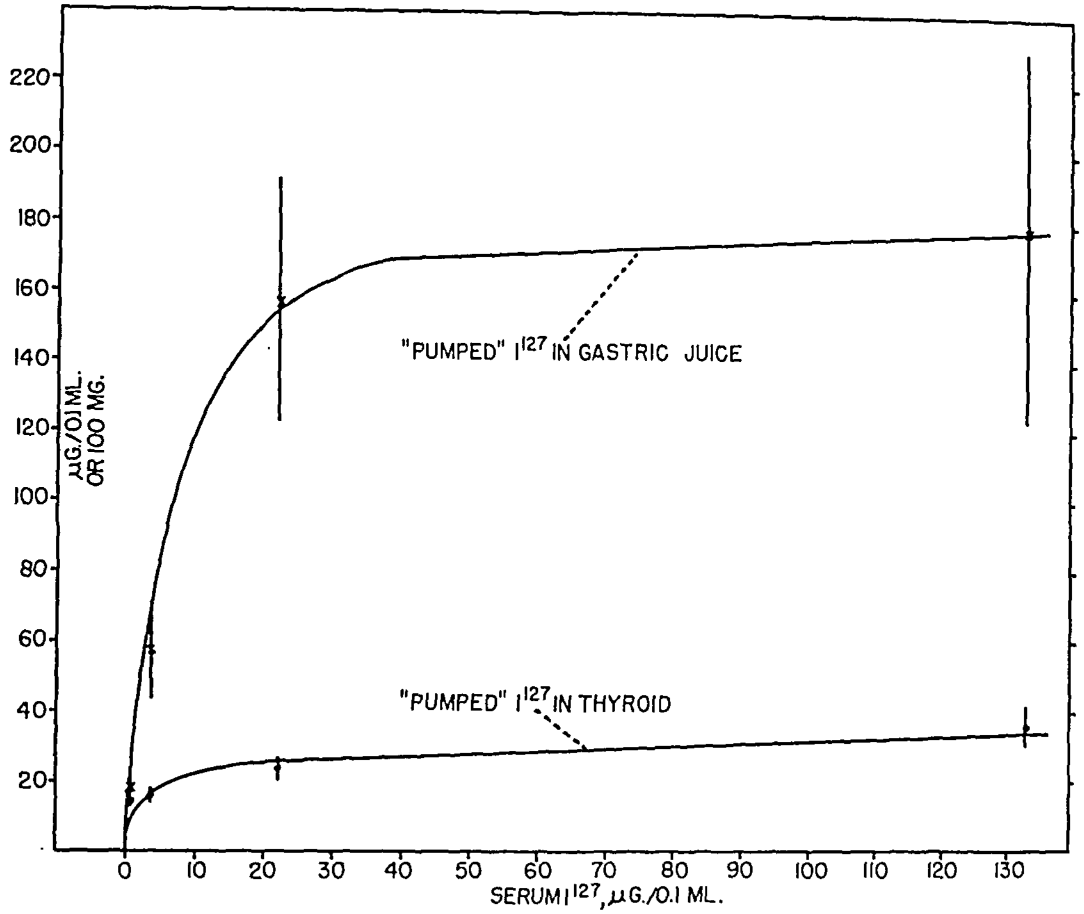

FIG. 1. Influence of serum iodine levels on the concentration of iodine in thyroid and gastric juice. Vertical lines: standard errors of the mean.

With graded doses of $NaClO_4$ similar results were obtained as with NaI^{127}: the highest dose lowered the J/S to 46% and the T/S to 11% of the control values (Table 3).

DISCUSSION

The rats fed a low iodine diet were probably secreting increased amounts of TSH, since their thyroid weights were significantly higher than those of the controls (Table 1). The high T/S of iodine deficient rats has been interpreted as the response of a sensitized iodide pump to normal or elevated levels of TSH (12, 13, 14). The failure of the gastric iodide pump to respond to iodine deficiency in part undoubtedly reflects the lack of effect of TSH on this mechanism, which was also evident in hypophysectomized rats given exogenous TSH (8). Since the gastric iodide pump did not even react to low iodine intake with the moderate activation observed in the case of the thyroidal pump of hypophysectomized rats fed a Remington diet for 19–20 days (13), the gastric wall apparently lacks an intrinsic regulator of its iodide pump like that postulated to exist in the thyroid (12, 13, 14).

Although the T/S and the J/S were approximately equal in rats fed a

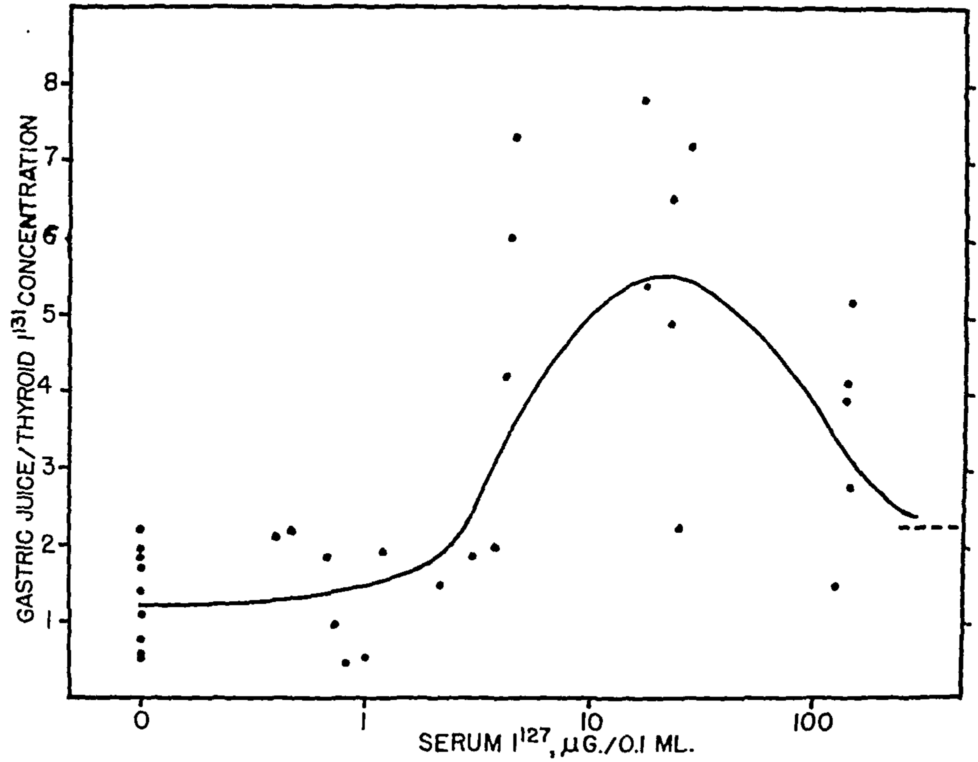

FIG. 2. Influence of serum iodine levels on the gastric juice/thyroid radioiodine concentration ratio. Each point stands for one rat. The line is drawn through the mean values observed in the groups of rats which were injected with NaI^{127} at the dose levels shown in Table 2.

diet containing adequate amounts of iodine and injected only with a tracer dose of I^{131}, loading with I^{127} showed that gastric juice has a much higher capacity for iodide than does the thyroid (Table 2, Fig. 1). This higher capacity, of course, may be entirely due to the fact that a known site of iodide concentration in the stomach, its secretion, was compared with the entire thyroid, which undoubtedly includes compartments in which iodide is not concentrated. An increase in the capacity of the thyroid for iodide is elicited by thyrotrophic stimulation, but this is accompanied by a commensurate increase of the T/S at physiologic serum iodide levels (15, 16). The activated thyroid thereby differs from the stomach. In the analysis of

TABLE 3. EFFECT OF $NaClO_4$ ON THYROIDAL AND GASTRIC IODIDE PUMPS IN RATS FED A REMINGTON DIET WITH IODINE (1 MG./KG.) ADDED

No. rats	Body wt., gm.	Dose of $NaClO_4$, μM	T/S	J/S
9	247 ± 7*	0	31.1 ± 4.4	36.4 ± 4.3
6	252 ± 4	0.308	17.4 ± 2.6	27.7 ± 9.1
6	242 ± 5	3.08	3.3 ± 0.6	16.7 ± 2.4

* Mean ± standard error.

their findings, Wollman and Scow (16) concluded that TSH affects the thyroidal iodide pump by means other than enhancement of the affinity of the transport carrier for its substrate, since it does not alter the K_M.[2] Comparison of the behavior of gastric and thyroidal iodide transport systems under conditions of loading with their substrate (Table 2) shows that the J/S declines less steeply with increasing serum iodide levels than does the T/S. A log-log plot of the data demonstrates that the K_M for the gastric iodide transport system is around 3 μg. of serum I^{127}/0.1 ml. vs. 1 μg./0.1 ml. for the thyroidal pump. This indicates that the gastric pump actually has a *lower* affinity for its substrate than that of the thyroid, which accounts for the fact that J/S and T/S are equal at physiologic serum I^{127} levels even though the capacity of the gastric juice for iodide is higher.

Earlier studies (18, 19) on dogs have also shown that the gastric iodide pump can be saturated with iodide. However, in the dog J/S values are depressed to 1 or below when the serum I^{127} reaches 3 mM/l. (=38.1 μg. /0.1 ml.) (19), whereas in the rat the J/S is still significantly above 1 when the serum I^{127} levels are four times as high.

It is not known whether the stomach of the rat concentrates perchlorate. The thyroid of several species has been found not to concentrate inhibitors of iodide transport such as thiocyanate or perchlorate (17, 20, 21). Freinkel and Ingbar (21) have therefore postulated that two carriers make up the thyroidal iodide pump: the first accepts competing anions as well as iodide, the second is specific for iodide. A similar situation may exist in the stomach wall, or gastric iodide transport may be accomplished by a single carrier. In any event, the T/S is more readily depressed than the J/S whether graded doses of I^{127} or perchlorate are given. This appears to indicate that the reason for the observed kinetic difference between the gastric and the thyroidal iodide pump resides in sites to which both iodide and perchlorate attach. It is also evident (compare Tables 2 and 3) that perchlorate is about 10 times as effective as iodide in depressing the J/S. The same is true for the T/S, which confirms earlier findings (2). Apparently perchlorate has a greater affinity for or easier access to the sites on which it acts when it inhibits thyroidal or gastric iodide transport.

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[2] The K_M, which is inversely proportionate to the affinity of the pump for its substrate, is the serum iodide concentration which results in a T/S or J/S that is one-half of the difference between the value obtained without loading with iodide and the value observed when the pump is completely blocked.

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OBSERVATIONS CONCERNING THE HETEROGENEITY OF OVINE THYROGLOBULIN[1]

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ABSTRACT

Preparations of thyroglobulin have been made by salting-out techniques from the thyroid gland of a single sheep, labeled with I^{131} *in vivo*, from multiple thyroid glands labeled *in vitro*, and from multiple unlabeled glands. Despite electrophoretic and ultracentrifugal homogeneity, such preparations displayed evidence of heterogeneity, as assessed by their behavior during ion-exchange chromatography, ultraviolet absorption spectra, rate of turnover of I^{131}, and amino acid content.

THE thyroid gland is an organ specialized to perform two principal biosynthetic functions. Great attention has been directed toward the first, *i.e.*, the formation of iodinated amino acids capable of exerting characteristic metabolic effects at distant sites within the organism. However, the second major biosynthetic function of the gland, the synthesis of a specific protein framework within which these iodinations may occur, has been largely overlooked. Yet, there is evidence that several of the characteristics of this protein may contribute greatly to normal hormonal economy. Although *in vivo* iodination of tyrosyl groups within proteins may occur in organs other than the thyroid gland, these extrathyroidal iodoproteins do not contain hormonally active iodinated amino acids (1–3) The demonstration that the hormonally active iodothyronines (as well as their iodotyrosyl precursors) are situated as end groups within the thyroglobulin molecule (4) suggests that the configurational characteristics of the thyroglobulin molecule may somehow facilitate their formation. In addition, the large molecular size of thyroglobulin seems certain to make the task of the thyroid gland in maintaining a large store of hormone more

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easily accomplished, and further, the capacity of thyroglobulin to hold thyroxine in a loose surface bond may afford some degree of regulation over the rate of hormonal release (5).

For these reasons, it seemed possible that a study of the synthesis of thyroglobulin might ultimately contribute to a better integrated understanding of the function of the normal and pathological thyroid gland. Before undertaking such a study, however, it seemed advisable to scrutinize more carefully the properties of the specific protein moiety in question.

Several unique characteristics of thyroglobulin, including its high concentration in a readily available tissue, its apparent relative ease of purification, and its large molecular size, have caused it to be employed as a model for the study of protein behavior in a number of systems, physicochemical as well as immunological (6–9). As a result, many of the physical constants and certain aspects of the chemical composition of thyroglobulin have been evaluated, and these in turn have served as criteria for assessing a number of methods for its isolation. Relatively recently, it has been shown that salting out techniques are capable of providing, from glandular extracts, preparations of apparently undenatured protein which have the physical and chemical characteristics of thyroglobulin (10). Although such preparations display electrophoretic and ultra-centrifugal homogeneity, they seem regularly to consist of several distinct moieties, as judged by solubility in salt solutions of high ionic strength (10). Attempts to assess the homogeneity of such preparations by other criteria, and to isolate the heterogeneous components, if possible, constitute the substance of the present report.

METHODS

Preparation of Thyroglobulin: Several batches of thyroglobulin were prepared from extracts of sheep thyroid glands by the method of Derrien, Michel, and Roche, employing ammonium sulfate as precipitating agent (10). Final preparations were extensively dialyzed against phosphate buffer, 0.01 *M*, pH 6.8, and were divided into aliquots which were frozen for use in individual experiments. This method of storing the protein was selected in view of the earlier demonstration that thyroglobulin loses its solubility after lyophilization (6).

The following batches of thyroglobulin were prepared:

Batches 1, 2, and 3: Thyroglobulin was obtained from pooled glands of several freshly killed sheep.

Batches 4 and 5: Twelve thyroid glands were obtained at the abattoir from freshly killed sheep. These were transported to the laboratory under refrigeration. Two glands were quickly sliced with a Stadie-Riggs microtome. Slices were distributed among a number of incubation flasks containing Krebs-Ringer-phosphate medium and a total of 500 μc. of inorganic I^{131}. After several hours of incubation at 38° C in an atmosphere of 100% oxygen, slices were removed, blotted, and pooled with slices which had been prepared from the remaining ten thyroid glands. The saline extracts of these glands was used to prepare Batches 4 and 5.

Batch 6: A single sheep was injected subcutaneously with 2 mc. of inorganic I^{131}. Twenty-four hours later the sheep was killed with a captive-bolt killer. The thyroid was quickly removed, freed of fat and fascia, and sectioned with Stadie-Riggs microtome.

Ion Exchange Chromatography: Ion exchange chromatography of preparations of thyroglobulin was performed, employing DEAE-cellulose, prepared according to techniques described by Peterson and Sober (11). Columns 1.5 cm. in diameter and 15 cm. long were most commonly employed. DEAE-cellulose was equilibrated with buffer (phosphate 0.01 *M*, pH 6.8) by means of a batch technique, following which the column was poured. Columns were packed and flow maintained under pressures of approximately 4 to 10 cm. of mercury, using either compressed air or nitrogen. After packing, the column was washed with another 50 ml. of buffer. The solution of protein, after dialysis against buffer of the same pH and molarity, was applied to the column, usually in a volume of 5 to 10 ml. An additional 50 to 100 ml. of starting buffer was than allowed to wash the column, and the total column effluent was collected during this period. In no case did significant quantities of radioactivity or protein pass through the column during the washing period.

Several experiments were performed in order to define optimum conditions for gradient elution. Ultimately, a gradient to buffer containing phosphate 0.05 *M*, NaCl 0.20 *M*, pH 5.7 was chosen. This buffer was passed from a reservoir into a mixing chamber containing 250 ml. of the starting buffer continuously agitated by a magnetic stirrer. Buffer was then passed to the top of the column. Fractions were collected in an automatic (constant interval) fraction collector, the flow-rate being adjusted by regulating the air pressure applied to the buffer reservoir. Flow was adjusted to a rate of approximately 20 ml./hour, and one fraction was collected every 15 minutes. Under these conditions, a complete elution could readily be performed overnight. Because of small variations in rates of flow, the volume of the individual fractions varied slightly.

In three experiments, the recovery of protein and radioactivity from the column was assessed. This was performed by analysis of the area under the elution curves. In all instances, recovery of radioactivity and protein exceeded 90%.

Analysis of Radioiodinated Amino Acid Content of Eluted Fractions: Protein fractions were precipitated from their eluting buffer with ammonium sulfate, were redissolved in urea-ammonium chloride solution, pH 8.5, and were subjected to tryptic hydrolysis at 38° C for twenty-four hours. Whole hydrolysates were then chromatographed on Whatman No. 1 paper in both butanol-dioxane-ammonia and butanol-acetic acid-water systems, using inorganic iodide, mono- and diiodotyrosine, triiodothyronine, and thyroxine as carriers. Carrier substances were localized with palladium chloride, in the case of iodide, and with diazotized sulfanilic acid, in the case of the iodinated amino acids. Localization of radioiodinated amino acids was determined by radioautography and by scanning the chromatographic strips in a continuous-feed-strip scanner in circuit with a rectilinear recorder. Quantitative assessment of the radioactivity in individual peaks in the chromatogram was obtained by analysis of their area.

Immunologic Techniques: Antisera against sheep thyroglobulin were prepared in three rabbits as follows: Solutions of thyroglobulin were suspended in Freund's adjuvant (light liquid paraffin, lanolin, and ground, heat-killed tubercle bacilli) to a final concentration of 6.25 mg./ml. Rabbits were given two intramuscular injections of 1.0 ml. of antigen one week apart. Following a rest period of four weeks, rabbits were twice injected intravenously with 5 mg. of thyroglobulin as alum-precipitated protein. Antisera were harvested two weeks later.

Miscellaneous Techniques: Solutions of thyroglobulin (1.3 gm.%) were electrophoresed in the 2.0 cm. cell of the Perkin-Elmer apparatus following prolonged dialysis against 0.05 *M* phosphate buffer, pH 6.8. Filter paper electrophoresis was performed in Whatman No. 3 paper in veronal buffer, pH 8.6, ionic strength 0.05. Spectrophotometric measurements were performed in a Unicam spectrophotometer, employing 1.0 cm. quartz cells. Nitrogen analyses were performed by a modified micro-Kjeldahl method. Measurements of radioactivity were carried out under conditions of constant

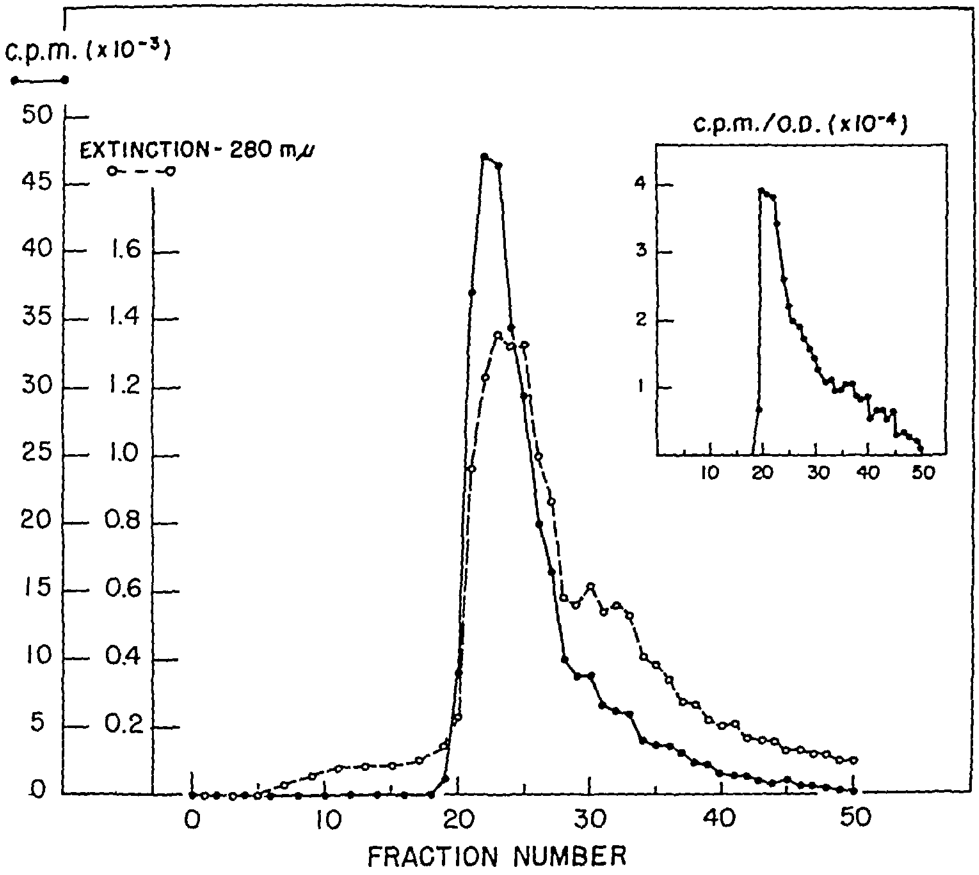

FIG. 1. Gradient elution of thyroglobulin from multiple sheep thyroid glands, labeled with I^{131} *in vitro,* from DEAE-cellulose resin. See text for chromatographic conditions.

geometry in a well-type scintillation counter. Sufficient counts were obtained to reduce the probable error of counting to less than 1%. Analyses for stable iodine were performed by the method of Barker, Humphrey, and Soley (12).[3] Proteins were analyzed for amino acid content by the technique of Moore and Stein (13), following hydrolysis in 6N HCl.

RESULTS

Batch 4 consisted of thyroglobulin extracted from twelve sheep thyroid glands, of which two had been allowed to accumulate I^{131} *in vitro* during an incubation period of several hours. Ion exchange chromatography of Batch 4 produced results depicted in Figure 1. Two principal findings were noted. First, the curve of protein elution was asymmetrical, displaying a rather sharp peak, followed by a distinct shoulder and tail. Second, fractions associated with the primary protein peak contained considerably more radioactivity than fractions in the shoulder and tail of the elution curve. Whereas proteins in the shoulder and tail of the elution curve contained approximately 40% of the ultraviolet absorbing moieties recovered from the column, they contained only 20% of the radioactivity. Calculation of the ratio of radioactivity to extinction at 280 mμ. for successive fractions of the elution curve revealed that peak ratio was achieved in the first fraction which contained appreciable protein. The ratio remained virtually constant in the next two fractions and then declined progressively, varying approximately seven-fold between the peak and the latest-eluting fractions.

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[3] Performed by Bioscience Laboratories, 231 South Carmelina Avenue, Los Angeles 64, California.

Consecutive fractions in the elution curve were mixed to form several pools (20–22, 23–25, 26–30, 31–36, 37–47) in which ultraviolet absorption spectra were determined. Results obtained in fractions 20–22 and 37–47 are illustrated in Figure 2. Maximum differences in absorption spectra were observed in pooled fractions from the extreme ends (20–22 and 37–47) of the elution curve (Fig. 2). The spectral curve shifted progressively from that displayed by fractions 20–22 toward that displayed by fractions 37–47 as pools from successive portions of the elution curve were examined. Early fractions displayed a distinctly lesser absorption in the 250–255 and in the 300–310 mμ. range than did fractions eluted late. Early fractions also displayed a distinctly sharper absorption peak at precisely 280 mμ. The spectrum of pooled fractions 37–47 revealed a broak peak which was almost flat between 278 and 282 mμ. Differences among the various pools diminished as the pH at which observations were made was increased. Maximum changes in absorption during change in pH occurred in the 245–255 mμ.

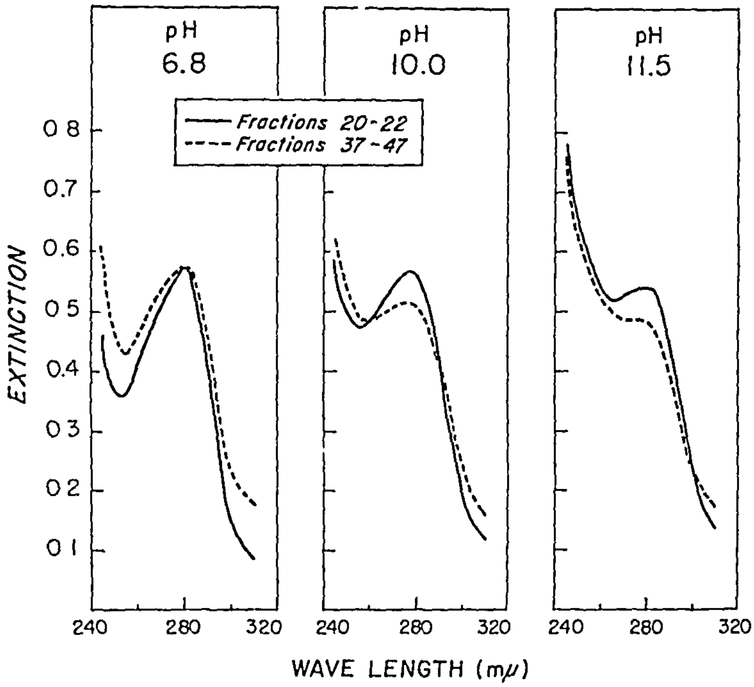

FIG. 2. Ultraviolet absorption spectra of pooled fractions of thyroglobulin from the elution curve shown in Figure 1.

ranges, were most marked when the pH was increased from 10.0 to 11.5, and were of greatest magnitude in pooled fractions 20–22.

Batch 5, representing a second preparation of thyroglobulin from the same saline extract as that which yielded Batch 4, gave entirely identical results with those obtained in Batch 4, both as regards the shape of the elution curve for radioactivity and for protein and as regards spectral absorption curves.

Pools were made of fractions in the early and in the late portion of the elution curve of Batch 5. These were dialyzed against 0.01 *M* phosphate buffer, pH 6.8, and were subjected to ultracentrifugal analysis (Fig. 3).

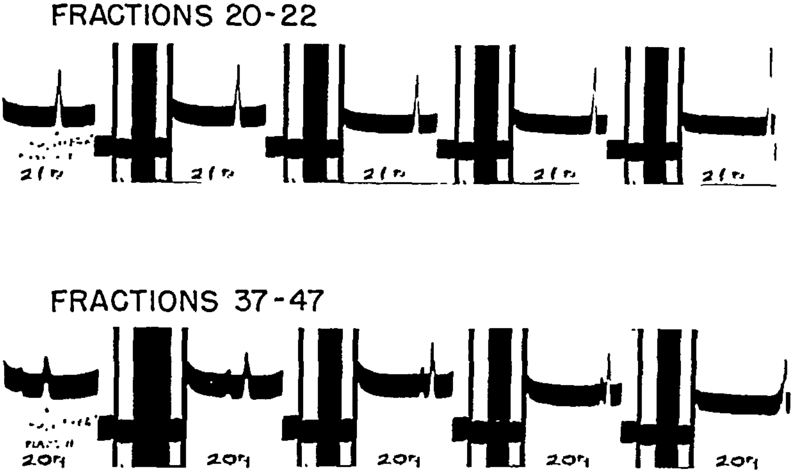

FIG. 3. Ultracentrifugal analyses of pooled fractions of thyroglobulin from the elution curve shown in Figure 1.

Except for a small, rapidly-sedimenting moiety in the late-eluting fractions, both fractions appeared homogeneous. Calculated values for $S_{20,w}$ were 19.2 and 19.7, respectively, for the early and late-eluting fractions. No moieties displaying less rapid sedimentation were seen.

When subjected to chromatography on the DEAE-cellulose column, Batch 2 of unlabelled thyroglobulin displayed an asymmetrical elution curve similar to that observed with Batches 4 and 5 (Fig. 4). Fractions 18–20 and 35–41 were dialyzed as separate pools against 0.01 *M* phosphate buffer, pH 8.6, and following dialysis, their absorption at 280 mμ. was determined. Known volumes of the two pools were combined into a single pool which was then chromatographed on the exchange column. During elution, two peaks appeared, corresponding very closely to the position of the fractions taken from the original elution curve, when correction was made for variation in the volume of the individual fractions. Calculated values revealed very good agreement between the quantity of U-V absorbing material recovered in each of the two peaks and the quantities applied from the corresponding portions of the original elution curve.

Batch 6, prepared from a single sheep thyroid gland, labelled with I^{131} *in vivo,* was chromatographed on the DEAE-cellulose column under standard conditions. Results are illustrated in Figure 5. The elution pattern obtained was virtually identical with that seen in Batches 4 and 5. Both asymmetry of the protein elution curve and non-uniform labelling were seen.

Differences in absorption spectra between early- and late-eluting portions of the curve in Batch 6 were the same as those described for Batches 4 and 5, preparations which contained thyroglobulin from the glands of multiple animals.

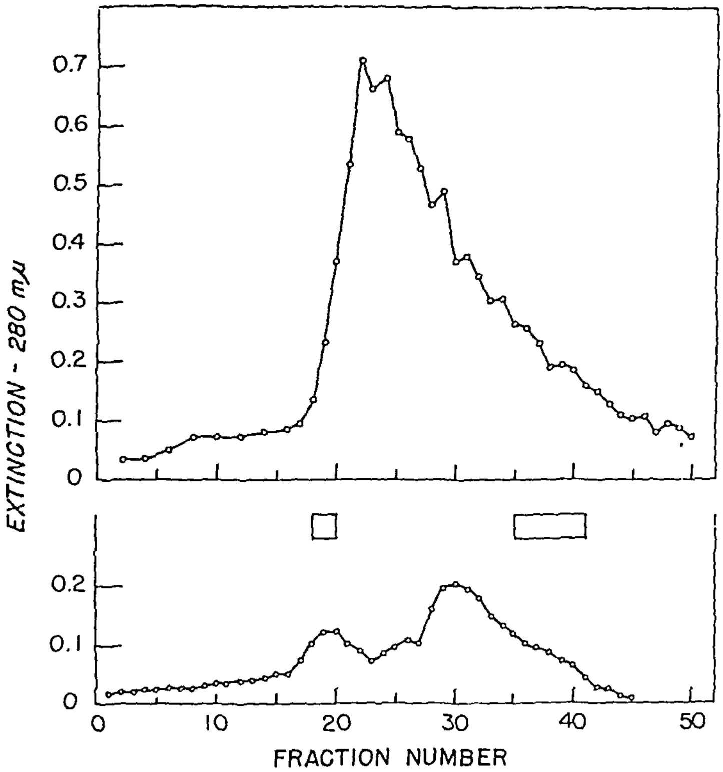

FIG. 4. Gradient elution of unlabeled thyroglobulin from multiple sheep thyroid glands. Fractions in the area indicated by the two rectangles were pooled and rechromatographed, with the results shown in the lower elution curve.

Tryptic hydrolysis and paper chromatography of alternate fractions of the entire elution curve revealed that each fraction contained iodide, mono- and diiodotyrosine, thyroxine and triiodothyronine, as well as other unidentified components. Among the several fractions, the relative proportion of iodinated amino acids of each type did not differ significantly.

Stable iodine analyses were performed on Fractions 21 and 31 of the elution curve of Batch 6. Iodine/nitrogen ratios were 28.4 μg./mg. in Fraction 21 and 26.6 μg./mg. in Fraction 31. Specific activity of iodine in Fraction 21 was calculated to be four times as great as that in Fraction 31. Similar results were obtained when iodine and nitrogen analyses were obtained in early- (28.7 μg./mg.) and late-eluting (31.2 μg./mg.) fractions of Batch 3.

Amino acid analysis of early- and late-eluting fractions from Batch 6 revealed small differences in the percentage composition of a number of amino acids (Table 1). Marked differences in two respects were observed. First, early eluting fractions contained considerably more valine, and second, late-eluting fractions contained a component which was completely absent from early-eluting fractions. This component has been tentatively identified as a hexosamine. The content of arginine was not determined in early-eluting fractions, as tubes containing that portion of the amino acid elution pattern were lost.

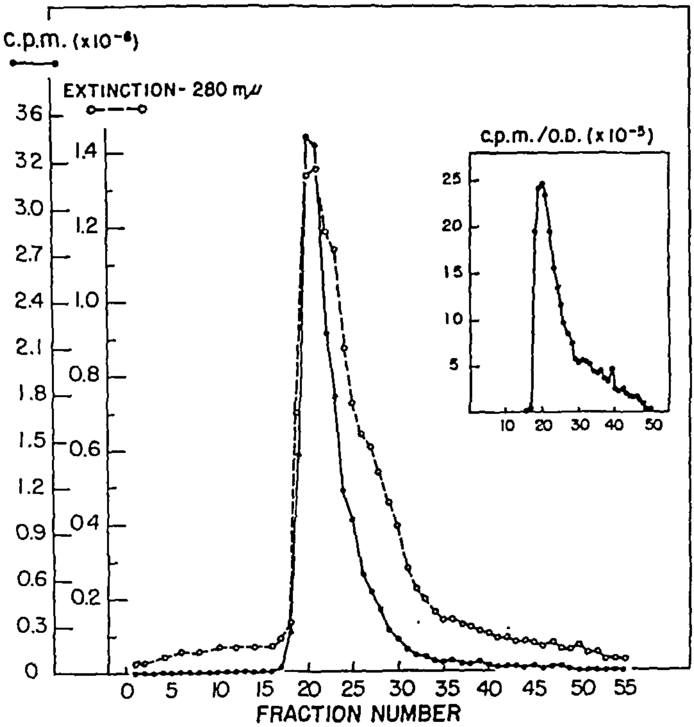

FIG. 5. Gradient elution of thyroglobulin from a single sheep thyroid gland, labeled with I^{131} *in vivo*, from DEAE-cellulose resin. Chromatographic conditions the same as those in Figure 1.

Study of the immunologic complexity of the whole thyroglobulin-antithyroglobulin system was performed by the Ouchterlony agar plate-diffusion technique (14). In this system, two distinct bands were seen in the zone of interaction between antiserum and whole, unfractionated thyroglobulin (the original antigen). The same two bands were found in both early- and late-eluting fractions from Batches 3 and 6. A small band was also seen when antiserum was allowed to interact with sheep serum, but this band was not confluent with those obtained during the interaction with thyroglobulin.

TABLE 1. AMINO ACID CONTENT OF EARLY- AND LATE-ELUTING FRACTIONS OF OVINE THYROGLOBULIN

	I Fractions 18–20*	II Fractions 28–31	III Fractions 28–31
Aspartic	7.8	9.0	8.4
Threonine	4.8	5.7	5.3
Serine	7.6	8.2	7.6
Glutamic	15.1	16.6	15.5
Proline	7.3	7.5	7.0
Glycine	5.0	5.3	4.9
Alanine	6.5	6.0	5.5
Cystine	1.1	0.9	0.8
Valine	9.5	5.7	5.3
Methionine	1.2	1.2	1.1
Isoleucine	2.6	2.6	2.4
Leucine	11.7	10.8	10.1
Hexosamine	0.0	1.9	1.8
Tyrosine	3.3	3.2	3.0
Phenylalanine	7.8	7.0	6.5
Lysine	4.2	3.5	3.2
Ammonia	2.2	2.5	2.3
Histidine	2.4	2.4	2.2
Arginine	—	—	7.1

* Tubes of Moore and Stein effluent containing arginine lost. Therefore, for purposes of comparison to each other, values in fractions 18–20 and 28–31 (Columns I and II) calculated on basis of content, exclusive of arginine. For purposes of comparison with other values in the literature, Column III presents values for fractions 28–31, including arginine.

DISCUSSION

The initial observation of inhomogeneity and non-uniform labeling in the thyroglobulin extracted from a pool containing the glands of several animals suggested that, despite its electrophoretic homogeneity, this preparation might be contaminated with another protein which was labeled little, if at all. This possibility seemed to be excluded by the ultracentrifugal experiments. These demonstrated that pools of both highly-labeled and slightly-labeled fractions had similar sedimentation constants, both being in the characteristic range for whole thyroglobulin (6, 8, 9). Even if it had contained no radioiodine at all, the small rapidly-sedimenting moiety found in the slightly-labeled fractions could not, by virtue of its low concentration, have accounted for the low I^{131}/N ratio of these fractions. Ultracentrifugal observations also served to exclude partial denaturation or hydrolysis of the protein as the cause of the apparent inhomogeneity, since neither pool displayed the characteristic alterations in sedimentation behavior which occur under these conditions (15, 16).

Further possible explanations were assessed in experiments performed with thyroglobulin prepared from a single sheep gland labeled *in vivo*. The striking similarity between the elution curves in this preparation and in thyroglobulin prepared from multiple glands served to exclude animal to animal variation as a source of inhomogeneity.

Another possibility was assessed by means of the material labeled *in vivo*.

It has been reported that during *in vitro* incubation of thyroid slices in media containing inorganic I^{131}, the principal radioiodinated products are iodotyrosines, while iodothyronines are labeled little if at all (17). Possibly, *preparations of thyroglobulin might contain a mixture of proteins, similar* in most respects, but varying markedly in their relative content of iodotyrosines and iodothyronines. In tissue labeled *in vitro,* proteins rich in iodotyrosines would therefore be much more highly labeled than proteins containing predominantly iodothyronines. Furthermore, the pK's of the iodotyrosines are higher than those of the iodothyronines and proteins rich in the former might be expected to elute from the column at a higher pH, *i.e.*, earlier in the elution curve. These considerations might explain the findings obtained during chromatography of thyroglobulin labeled *in vitro.*

During *in vivo* labeling, radioiodinated thyroxine and triiodothyronine were formed. It was possible to demonstrate by paper chromatography that the fractions obtained throughout the elution curve all contained iodotyrosines and iodothyronines in approximately the same proportions. This did not prove conclusively, however, that the stable iodinated amino acids in all fractions were quantitatively and qualitatively the same, since the radioactive components found in late-eluting fractions may merely have been portions of the early-eluting fractions which trailed off the column. Nevertheless, I^{127}/N ratios in the two extremes of the elution curve were virtually identical, indicating that, whatever their chemical nature, iodinated moieties in the late-eluting fractions were turning over very slowly.

It also seemed possible that the curve of protein elution might represent the behavior of a single species of protein, and that the asymmetry observed was merely a function of the chromatographic system. This possibility was rendered unlikely by experiments performed with unlabeled thyroglobulin, Batch 3. Fractions from the two extremes of the elution curve were pooled and rechromatographed. During rechromatography, two peaks were obtained. Had asymmetry of the elution pattern resulted merely from trailing of a homogeneous protein, a single, asymmetrical peak, similar to the first, would have been expected during rechromatography. The appearance of two peaks, however, suggested the presence of two distinct groups of components, chromatographing independently.

This experiment, employing unlabeled thyroglobulin, further indicated that the asymmetry observed in previous experiments could not be explained by radiation damage to the protein.

As a final criterion of heterogeneity, amino acid analyses were performed on fractions from the extreme portions of the elution curve of thyroglobulin from a single gland (Batch 6). Since hydrolysis of protein was carried out in 6N HCl, analyses for tryptophane could not be obtained. Small differences in the percentage composition of a number of other amino acids were seen, and striking differences were found in the content of valine and of another component, probably a hexosamine. The latter component was found only in the late-eluting fractions. In general, values for the various amino acids in both groups of proteins agreed well with those previously reported for the thyroglobulins of other species, studied by a number of different techniques (9, 18). One possible discrepancy between the present values and those reported earlier was found in the case of arginine. This has been reported to be the preponderant amino acid in thyroglobulin, values as high as 12.7% having been noted (9). In the present study, arginine constituted but 7% by weight of the proteins in the late-eluting fractions. Unfortunately, loss of the appropriate samples prevented analysis for arginine in the earlier fractions. It is apparent, however, that the arginine content of these fractions would necessarily have been very high in order for the average arginine content of the entire batch to have been 12%. Alternatively, the low arginine content of the late-eluting fractions might be explained by the relative resistance of some of the arginine bonds in thyroglobulin to acid hydrolysis (19). A further difference between the present and previously noted values was seen in the content of valine. Present values of from 5.7 to 9.5%, obtained in the sheep, are far higher than those reported for the porcine protein (9).

Considered together, the present observations thus indicate that even within the thyroid gland of a single animal, thyroglobulin is not entirely homogeneous, but rather may be a complex mixture of closely related proteins.[4] Preliminary studies have revealed similar complexity in thyroglobulin extracted from human material (20). The precise relationship of the present findings to the earlier demonstration that thyroglobulin appears to be composed of three moieties, as judged by solubility characteristics, remains unclear. Although many of the observations herein presented were made in fractions expected to display maximum differences, it seems likely that between these extremes there lies a spectrum of closely related proteins. It is noteworthy that the present techniques, although effective in separating these moieties at least partially, did not resolve the apparent immunological complexity herein demonstrated for sheep thyroglobulin, and previously noted in human material (21).

The physiological significance of these findings is obscure. However, one possible explanation for heterogeneity of thyroglobulin arises from the observation that within a single thyroid gland, individual follicles may show differing degrees of activity (22, 23). It seems possible, therefore, that the different thyroglobulins noted in the present study may be the products of follicles of varying function. Extension of studies to include the thyroglobulin of normal and abnormal human or animal glands might clarify this relationship. In addition, the function, if any, of protein-bound carbohydrate, *including hexosamine, in which the thyroid gland is relatively* rich (18, 23–25), seems to merit attention, especially since hexosamine was found only in fractions of thyroglobulin whose iodine was turning over slowly.

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[4] Attempts to correlate the chromatographic behavior of the various fractions with their amino acid and carbohydrate contents, and with their spectral absorption curves, are beyond the scope of the present communication, and will be presented elsewhere.

Finally, it is of interest that differences in rates of iodination are accompanied by variations in the chemical composition or configuration of thyroglobulin. Recent studies suggest that the synthesis of iodinated amino acids *in vivo* cannot be divorced from the synthesis of thyroidal protein, and that the two processes are either related, or at least occur *pari passu* (20). Relevant to these observations, is the demonstration of abnormal iodoproteins in the plasma of some patients with thyroidal carcinoma (26), congenital goiter (27), or adult non-toxic goiter (28). Abnormal thyroglobulins have also been found in carcinomatous thyroid tissue (29) In these abnormal proteins, hormonally active iodinated amino acids are not found. Thus, the relation between abnormal protein synthesis and disordered hormonal biogenesis would seem to merit further investigation. Such studies are now in progress.

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THE EFFECT OF VARIOUS THYROXINE ANALOGUES ON SUPPRESSION OF I^{131} UPTAKE BY THE RAT THYROID[1]

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ABSTRACT

Forty-five different thyroxine analogues have been compared to *l*-thyroxine for their ability to decrease the thyroid collection of I^{131} in the rat. On a weight basis only 3,5,3′-triiodo-*l*-thyronine was found more effective than *l*-thyroxine. Compounds containing iodine were all capable of producing some inhibition providing the dosage was adequate.

THE dose of thyroid hormone, within limits, can be directly related to thyroidal uptake of I^{131} in experimental animals. This method has been used in the present report to assay the relative effectiveness of thyroxine analogues in blocking uptake of radioiodine in rats.

METHODS AND MATERIALS

Approximately 2,500 male rats of the Sprague-Dawley strain (Charles-River Breeding Laboratories, Boston, Massachusetts) ranging in weight between 75–125 gm. were used in this study. They were selected so that the average body weight of each group was essentially identical within each separate experiment. At the start of each study animals were placed on a low iodine diet (1) and given 0.1% thiouracil in drinking water. After 5 days of this treatment the water containing thiouracil was replaced with ordinary tap water which was continued to the termination of the experiment. Twenty-four hours after the thiouracil was stopped, daily subcutaneous injections of the test analogues were started. These were administered for 5 days and 24 hours after the last injection all animals were given a single intraperitoneal injection of 0.1 μc of I^{131}. These animals along with a control group of a similar number of uninjected rats and a group maintained on regular laboratory diet were autopsied 24 hours after the administration of I^{131}. At autopsy the thyroids were removed, dissected free of extraneous tissue, weighed on a Roller-Smith balance and placed in 5 cc. vials containing a standard volume of Bouin's solution. Radioactivity was determined using a well-type scintillation counter and the thyroid uptake of I^{131} calculated using an appropriate standard.

In any given experiment 3 doses of each analogue (dissolved as previously described (2) and injected in a volume of 1 cc.) were assayed with 5–10 animals per group. Calculations for relative potency compared with *l*-thyroxine were made as follows. Actual data obtained from an assay were plotted on semi-log paper and the dose of the analogue

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TABLE 1. RELATIVE INHIBITORY ACTIVITY (R.A.) OF VARIOUS ANALOGUES IN BLOCKING UPTAKE OF I^{131} BY THE RAT THYROID

Compound	RA
Thyronine series	
l-thyroxine	100
d-thyroxine	50
dl-thyroxine	40
3,5,3',5'-tetrachloro-dl-thyronine	<1.5
3,5,3'-triiodo-l-thyronine	300
3,3',5'-triiodo-dl-thyronine	75
3,5-diiodo-l-thyronine	37
3,3'-diiodo-dl-thyronine	100
3-iodo-dl-thyronine	50
1-nitro-5-(3,5-dinitrothyromethylene) hydantoin, methyl ether	<3
Butyric series	
3,5,3',5'-tetraiodothyrobutyric acid	30
3,5,3'-triiodothyrobutyric acid	75
3,5-diiodothyrobutyric acid	60
Acrylic series	
3,5,3',5'-tetraiodothyroacrylic acid	50
Propionic series	
3,5,3',5'-tetraiodothyropropionic acid	60
3,5,3'-triiodothyropropionic acid	60
3,3',5'-triiodothyropropionic acid	50
3,5-diiodothyropropionic acid	16
3,5-diiodothyromethylmalonic acid, methyl ether, diethyl ester	20
3,3'-diiodothyropropionic acid	25
3-iodothyropropionic acid, methyl ether	17
3-aminothyropropionic acid	Inactive
3-aminothyropropionic acid, methyl ether	<0.3
3-aminothyropropionic acid, methyl ether, ethyl ester	Inactive
3,5-diaminothyromethylmalonic acid, methyl ether, diethyl ester	Inactive
3-nitrothyroacrylic acid, methyl ether	<1.5
3-nitrothyroacrylic acid, methyl ether, ethyl ester	Inactive
3,5-dinitrothyromethylenemalonic acid, methyl ether, diethyl ester	<1.5
Acetic series	
3,5,3',5'-tetraiodothyroacetic acid	75
3,5,3'-triiodothyroacetic acid	75
3,3',5'-triiodothyroacetic acid	15
3,5,3'-triiodothyroacetic acid, methyl ether	37
3,5-diiodothyroacetic acid	18
3,5-diiodothyroacetic acid, methyl ether	50
3,3'-diiodothyroacetic acid	25
3-iodothyroacetic acid	33
3,5-diaminothyroacetic acid, methyl ether	7.5
3,5-dinitrothyroacetic acid, methyl ether	Inactive
3,5,3'-trinitrothyroacetic acid, methyl ether, ethyl ester	Inactive
Formic series	
3,5,3',5'-tetraiodothyroformic acid	10
3,5,3'-triiodothyroformic acid	15
3,5-diiodothyroformic acid, methyl ether, methyl ester	15
3,5,3'-triaminothyroformic acid, methyl ether, methyl ester	Inactive
3,5-diaminothyroformic acid, methyl ether, methyl ester	Inactive
3,5-dinitrothyroformic acid, methyl ether	Inactive
3,5-dinitrothyroformic acid, methyl ether, methyl ester	Inactive

necessary to produce a control (untreated animals on regular diet) uptake of radioiodine read off from the graph. The dose of *l*-thyroxine necessary to produce this effect was consistently between 10 and 15 μg. This figure was arbitrarily assigned a value of 100, and calculations were made for the analogues to give a relative potency in terms of *l*-thyroxine. These values are shown in Table 1. A compound showing no ability to lower I^{131} uptake even in doses of 500 μg. daily is reported as inactive.

RESULTS AND DISCUSSION

The data in Table 1 were obtained using 46 different compounds. Most of these compounds were also tested at the same time for their ability to produce metamorphosis of the tadpole as has been reported (2). Similarly, the determination of the antigoitrogenic and calorigenic activities of the analogues have been determined in rats. These latter studies are reported in the present issue of *Endocrinology*. The reader may find the structural formulae of the compounds in Table 1 by referring to the paper of Stasilli *et al.*

It is apparent from Table 1 that under the conditions used only 3,5,3′-triiodo-*l*-thyronine is more effective on a weight basis than *l*-thyroxine in blocking the uptake of I^{131} by the rat thyroid. Whether other compounds would prove to be more effective at times other than 24 hours after I^{131} was not determined. It is of interest that only one analogue showed the same relative inhibitory activity as *l*-thyroxine.

It seems rather unlikely that the number of atoms of iodine in the molecule is the chief factor involved in blocking thyroidal I^{131} collection. For example, *d*-thyroxine appears to be only one-half as effective as the *l*-isomer while *d*-*l*-thyroxine is no more active than the *d*-form. In the triiodinated compounds, as was found in tadpole metamorphosis (2), in general the 3,5,3′-analogues appear to be somewhat more effective than the 3,3′,5′ compounds. All analogues which contained iodine manifested various degrees of inhibitory effect on the uptake of I^{131} when given in adequate doses. On the other hand few non-halogen containing compounds showed evidence of inhibitory activity.

Other studies (2, 3) have pointed out the difficulties encountered in studies using thyroxine analogues. The major problems in obtaining valid data are the purity and stability of the compounds. The latter problem has been considered (3) in some detail and was realized when the present studies were done. However, as has been pointed out (3) degradation of some of these compounds does occur. How much and in what way the present results may have been altered by this degradation is at present not certain.

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THE *IN VITRO* METABOLISM OF THYROXINE, TRIIODOTHYRONINE AND THEIR ACETIC AND PROPIONIC ACID ANALOGUES[1]

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ABSTRACT

The metabolism of thyroxine, triiodothyronine and their acetic and propionic acid analogues has been studied by placing these I^{131} labelled substrates in buffered medium with or without added tissue. Spleen, kidney and liver slices, homogenates and fractions of homogenates were used. Samples of medium and/or homogenized slices, usually after three hours' incubation at 37° C, were chromatographed and quantitated by strip counting. The only product of deiodination detected was iodide. When thyroxine endogenously labelled with I^{131} was used, no 3, 5 diiodothyronine or other partially deiodinated thyronines or derivatives were formed, although activity was low and small amounts of other products could not be ruled out. Deiodination was inhibited by a nitrogen atmosphere, boiling, heavy metals, cyanide, 2 methyl 1, 4 naphthoquinone, a chelating agent (Versene), and estrogenic substances. No antithyroid substances, including iodide and propylthiouracil, had any effect; iodinated tyrosines and organic iodine compounds had no effect. All tetra and tri iodinated thyronines inhibited the deiodination of other tetra and tri iodinated thyronines, often commensurate with the degree of their own deiodination. Only one iodinated compound, other than these, was an effective inhibitor: N-butyl para hydroxy diiodobenzoate.

IN VIVO and *in vitro* studies of thyroxine and triiodothyronine have indicated several pathways of metabolism involving deiodination, decarboxylation and deamination. Gross and Leblond (1) gave thyroxine labelled with I^{131} to thyroidectomized mice and found radiotriiodothyronine (unknown 1) in the organs. The conversion of thyroxine to triiodothyronine by kidney slices has been demonstrated (2), although the chroma ographic differentiation of triiodothyronine from the acetic or propionic acid analogue of thyroxine has been questioned (3). Deiodination of radiothyroxine and radiotriiodothyronine by rat skeletal muscle homogenates (4) resulted in the formation of radioiodide and small amounts of other unidentified products; whereas, rat brain homogenates (3) formed significant amounts of the acetic and/or propionic acid analogues in addition to iodide, and, occasionally, small amounts of triiodothyronine. Rat kidney homogenate and mitochondria incubated with radiothyroxine did not result in the formation of triiodothyronine or iodide but rather in the acetic acid analogue (5). Similar studies with radiotriiodothyronine indicated that the acetic acid analogue was the product formed (5). Roche (6)

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[1] Presented at the 1958 Meeting of the American Goiter Association.

et al. have demonstrated that the kidneys of thyroidectomized rats treated with radiotriiodothyronine contain radiotriiodothyroacetic acid and (7, 8) that the pyruvic acid analogues are found in the urine and bile after the injection of radiothyroxine and radiotriiodothyronine. Taurog *et al.* (9) have indicated that conjugation of thyroxine with glucuronic acid occurs in the presence of liver slices.

Tissue deiodinating systems (2, 4) were inhibited by boiling. Anaerobic conditions (3, 4) did not affect the results but cyanide (2, 3), iodide, (2) and mercuric chloride (3) were effective inhibitors.

The present study on the metabolism by tissue preparations of thyroxine and triiodothyronine attempts to clarify the products formed, the effect of various substances added to the medium with particular emphasis on compounds known to affect the thyroid, enzyme inhibitors and other iodinated compounds, and the specificity of the enzyme system responsible for deiodination.

METHOD

Livers, spleens, muscle and kidneys were obtained from normal Sprague-Dawley male rats (200–250 gm.) killed by ether. Slices were cut freehand (0.5–1.5 mm.) and placed (200–300 mg.) in 2 ml. of modified Krebs-Ringer-phosphate solution ($CaCl_2$ $8\times10^{-4}M$) at pH 7.4 to which 2–3 μc. of I^{131} labelled (as obtained from Abbott Labs) *l*-thyroxine, 1–3,5,3′ triiodothyronine, tetraiodothyroacetic or propionic acid, 1–3,5,3′ triiodothyroacetic or propionic acid had been added. The tubes were incubated at 37° C with constant shaking for 3 hours. Substrate concentrations of $2.6\times10^{-5}M$ to $5.6\times10^{-2}M$ were obtained by adding the appropriate amount of carrier. Homogenates were similarly treated. Fractionation of tissue homogenized in 0.25*M* sucrose solution was accomplished by differential centrifugation (modified Hageboom). The following fractions, in a concentration of 400 mg. of tissue per 4 ml. were then treated as above: whole homogenate, nuclei and debris, mitochondria, microsomes, supernatant and a combination of mitochrondria, microsomes and supernatant. After incubation (slices were homogenized) an aliquot (20λ) was placed on paper and chromatographed in t-amyl alcohol saturated with 2*N* NH_4OH (descending) and/or n-butanol-acetic acid-water (78–10–12). In several experiments the five solvent systems noted below were used. Known carrier spots[2] were identified with ninhydrin or ceric sulfate-arsenious acid. The strips were exposed to x-ray film and counted on a strip counter (Nuclear-Chicago). Planimeter measurements were made for quantitation.

Thyroxine labelled endogenously with radioiodine was obtained by feeding rats propylthiouracil (0.3%) for 4 days and discontinuing it 18 hours prior to injecting 100 μc. of radioiodine. Twenty-four hours later the rats were sacrificed, thyroid glands removed, homogenized, hydrolyzed by pancreatin for 24 hours in K-R-P at pH 7.4 and extracted with butanol saturated with acidified water (pH 3.5). The extract was then evaporated, redissolved in a small amount of butanol and chromatographed in t-amyl alcohol saturated with 2*N* NH_4OH. The radio-thyroxine spot was eluted with butanol and acidified water, evaporated to dryness and redissolved in 50% propylene glycol. The iodine content of this material was $10^{-7}M$ as determined by a modified Barker technique. An aliquot of this endogenously labelled radiothyroxine was added to tubes with rat liver slices. At 6 hours a sample of the medium was placed on paper, the tissue was homogenized and placed on paper directly or extracted with butanol and acidified water. The extract was evaporated to dryness and redissolved in 0.5 cc. butanol and acidified water. Five solvent systems were used for chromatography: t-amyl alcohol-ammonium hydroxide, butanol-dioxane-ammonium hydroxide, collidine-water in an atmosphere of ammonia, butanol-acetic acid-water and butanol-ethanol-ammonium hydroxide. In one experiment the rats were injected with radioiodine without prior treatment with propylthiouracil. After incubation with pancreatin, the medium was directly chromatographed without extraction and the areas of thyroxine and triiodothyronine were cut out and placed directly in the control or liver tubes. At 4 hours the medium was placed on paper.

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[2] Thyroxine, 3,5,3′ triiodothyronine, and the propionic and acetic acid analogues were kindly supplied by Dr. Michael Barry of Warner-Chilcott Laboratories.

RESULTS

Deiodination of thyroxine and triiodothyronine and the products formed

The major portion of deiodination occurred within three hours and only minor changes occurred thereafter. Temperatures of 5° C completely inhibited and 50° C and 70° C reduced the amount of deiodination; 37° C seemed to be optimal. Table 1 illustrates the degree of deiodination of thyroxine and triiodothyronine when incubated with liver, spleen, or kidney slices. Liver and kidney (and muscle) were active whereas spleen was only slightly active. At three hours there was more deiodination of thyroxine than triiodothyronine (Table 1), indicating that thyroxine was more rapidly deiodinated.

TABLE 1. DEIODINATION OF L-THYROXINE AND L-3,5,3′-TRIIODOTHYRONINE BY RAT LIVER, KIDNEY, AND SPLEEN SLICES (250 MG./3 CC.) INCUBATED FOR 3 HOURS

	L-thyroxine 7.6×10^{-7}M*			L-3,5,3′-triiodothyronine 2×10^{-8}M		
	% total distributed as			% total distributed as		
	T4	I^-	O†	T3	I^-	O
Control	80.6	9.2	10.2	95.5	3.6	0.9
Liver	35.4	54.6	10.0	65.7	27.7	6.6
Spleen	64.6	28.9	6.5	86.6	10.5	2.9
Kidney (Cortex)	44.3	50.0	5.7	64.0	30.7	5.3

* Note that concentration of T4 is 35×T3.

† Represents radioactivity staying at the origin in t-amyl alcohol saturated with 2N NH_4OH. In occasional experiments there were traces of unknowns and material at the front.

Iodide, released from the deiodination of thyroxine and triiodothyronine, was present in the medium. Small amounts of material travelling at the front were not considered significant. As much as 10% (and, on one occasion, 20%) of the total radioactivity remained at the origin on chromatography. Although small amounts stayed at the origin from tubes without tissue, the amount at the origin increased markedly in the presence of tissue. This was presumably iodinated protein or thyroxine and protein complexed so as to be insoluble in t-amyl alcohol, though it was not hydrolyzed and rechromatographed. Unidentified compounds occurred sporadically, the most frequent one having an R_f, in t-amyl-ammonium hydroxide, between those of iodide and thyroxine. This unidentified material was present in the control tubes and diminished markedly after incubation with liver slices. When butanol-ethanol-ammonium hydroxide was used as a solvent, a radioactive spot just above the origin often appeared after incubation. Since this spot also appeared when radioactive iodine alone was chromatographed, it was felt to be an oxidized derivative of iodide. In some instances, chromatography of thyroxine labelled with radioiodine (Abbott) and incubated in control tubes for three hours without tissue would result in six distinct spots in t-amyl alcohol-2N NH_4OH while only two distinct spots (iodide and thyroxine) were noted in butanol-ethanol-2N NH_4OH. These spots of unknown compounds were all diminished in amount when the thyroxine was incubated with liver slices. A question arises as to whether these various spots represent compounds originally present or factitiously produced by the action of t-amyl alcohol. It is of interest that carrier thyroxine is more difficult to demonstrate by ninhydrin stain after chromatography with t-amyl alcohol solvent than with butanol-ethanol-ammonium hydroxide.

TABLE 2. ACTIVITY OF LIVER FRACTIONS IN DEIODINATION OF L-THYROXINE

Tissue	L-thyroxine 2.6×10^{-6}M		
	% of total distributed as		
	T4	I^-	O
Slices	45.8	34.2	20.0
Homogenate	41.0	47.3	11.7
Nuclei and debris	64.8	24.1	11 1
Mitochondria	39.0	42.7	18.3
Microsomes	74.9	20.8	4.3
Supernatant fluid	58.3	29.7	12.0
Mitochondria, microsomes and supernatant	32.2	56.0	11.8
Control	75.2	16.2	8.6

Localization of deiodination and the effects of anaerobiosis and boiling

Slices, homogenate, mitochondria, and the combination of mitochondria, microsomes and supernant were all active in the deiodination of thyroxine (Table 2). Nuclei and debris, microsomes and supernatant fluid were much less active indicating that the mitochondria was the most active of the cell fractions. Anaerobic conditions inhibited the deiodination of thyroxine by slices and mitochondria and of triiodothyronine by slices. Boiling ten minutes markedly reduced the deiodination of thyroxine by slices and mitochondria and of triiodothyronine by slices. Thirty minutes boiling of the homogenate did not affect the deiodination of thyroxine or triiodothyronine, although sixty minutes of boiling inhibited to a moderate extent.

Effect of substrate concentration

Increasing the concentration of *l*-thyroxine and *l*-triiodothyronine above physiological levels markedly reduced the per cent of radioactive material deiodinated by slices and homogenates. At 10^{-5} M the per cent deiodinated of both is so low as to be unmeasurable. At 10^{-7} M thyroxine is 80–90% deiodinated. Triiodothyronine at 10^{-8} M is only 50% deiodinated. There is, then a marked difference between the quantity of thyroxine and triiodothyronine which can be deiodinated by liver slices or homogenates during an equal period of time.

Inhibition by various compounds of deiodination

The deiodination of thyroxine and triiodothyronine by liver slices and homogenates is markedly inhibited by mercuric chloride and n-butyl parahydroxy, 3,5-diiodobenzoate (BHDB) (Table 3). Deiodination by mitochondria is not, however, affected by BHDB (Table 3). Less marked, but definite, was the inhibition by other heavy metals (cupric chloride, cobaltous sulfate), some phenols (resorcinol, estradiol), a quinone (2-methyl 1,4-naphthoquinone), a chelating agent (ethylene diamine tetra acetic acid), and an oxidative enzyme inhibitor (sodium cyanide, not charted). The results with hydroquinone and silver nitrate were difficult to interpret due to their effect on the chromatographic characteristics of iodine compounds (10). The estrogenic substances, estrone and estradiol, were effective inhibitors whereas corticosterone, hydrocortisone, desoxycorticosterone, testosterone and progesterone were ineffective. Reserpine and thyronine had no effect. Sodium azide may have had a slight inhibitory effect but the following compounds had no effect: compounds which affect thyroid function (iodide, Tapazole, propylthiouracil, sulfanilamide, potassium thiocyanate), other iodinated compounds (iodide, monoiodotyrosine, diiodotyrosine, 3,5-diiodothyronine, Teridax,[3] Priodax,[4] Telepaque[5]), a specific sulfhydryl group inhibitor (parachloromercuribenzoate).

The deiodination of acetic and propionic acid analogues of thyroxine and triiodothyronine

The deiodination of the acetic acid analogues of thyroxine and triiodothyronine (about 0–10%) by liver slices (Fig. 1) or muscle homogenates (Table 4) was much less than the deiodination of thyroxine (about 75–90%) and probably less than the deiodination of triiodothyronine (about 30%). The deiodination of propionic acid analogues of thyroxine and triiodothyronine (30–45%) by liver slices (Fig. 1), though less than the deiodination of thyroxine (80%), was about the same as triiodothyronine, whereas deiodination of propionic acid analogues by liver and muscle homogenates (Table 4) was about the same as the acetic acid analogues. It is of interest that triiodothyropropionic acid was slightly more deiodinated than tetriiodothyropropionic acid (Fig. 1), the only triiodo form of thyro-

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[3] β(5-hydroxy 2,4,6 triiodophenyl)-α-ethyl propionic acid.

[4] β(4-hydroxy 3,5 diiodophenyl)-α-phenyl propionic acid.

[5] β(3-amino 2,4,6 triiodophenyl)-α-ethyl propionic acid.

TABLE 3. DEIODINATION OF L-THYROXINE AND 1-3,5,3'-TRIIODOTHYRONINE BY RAT LIVER IN THE PRESENCE OF VARIOUS SUBSTANCES AT 10^{-3}M

Substance added	Mitochondria			Slices			Slices			Homogenate			Homogenate		
	% total thyroxine 6×10^{-8}M distributed as			% total thyroxine 7.1×10^{-7}M distributed as			% total triiodothyronine 6.5×10^{-7}M distributed as			% total thyroxine 7.1×10^{-7}M distributed as			% total triiodothyronine 6.5×10^{-7}M distributed as		
	T4	I^-	O	T4	I^-	O	T3	I^-	O	T4	I^-	O	T3	I^-	O
Control	10.6	77.5	11.9	13.3	80.2	6.5	56.3	40.7	3.0	25.0	65.0	10.0	49.2	40.2	10.8
$HgCl_2$	85.0	0.0*	5.0	80.6	9.2	10.2	77.8	16.1	6.1	67.5	28	4.2	77.9	17.8	4.3
$CuCl_2$	78.0	18.3	3.7	39.0	50.7	10.3	80.0	13.5	6.5	65.0	24.3	10.7	95.6	3.9	0.3
$CoSo_4$	68.4	15.7	15.9	42.3	47.0	10.7	75.4	20.0	4.6	73.0	14.5	12.5	87.4	8.4	4.2
Resorcinol	61.2	24.7	14.1	39.9	57.0	3.1	71.2	23.6	5.2	77.5	16.5	6.0	72.4	24.6	3.0
Estrone	82.4	9.2	8.4	48.0	41.6	10.4	92.1	6.8	1.1	76.5	20.0	3.5	94.5	5.5	0.0
Estradiol	—	—	—	56.1	34.3	9.6	94.5	5.5	0	78.5	13.0	8.5	93.6	6.4	0.0
Corticosterone		—	—	20.0	75.8	4.2	53.0	34.2	12.2	28.0	71.0	1.0	63.8	22.8	3.4
DOCA	—		—	21.4	72.9	5.7	57.4	40.7	1.9	—	—	—	60.7	30.9	8.4
Testosterone	—		—	5.2	85.7	9.1	64.2	25.9	9.9	33.0	61.5	5.5	67.5	27.9	4.6
Progesterone	—		—	8.6	89.6	1.8	68.3	25.5	6.2	27.5	66.0	6.5	64.2	32.9	2.9
$2CH_3$-1,4-Naphthoquinone	80.4	9.3	10.3	41.2	45.2	13.6	79.2	15.3	5.5	86.0	10.9	3.1	82.4	13.4	4.2
EDTA†	81.2	15.2	3.6	50.6	46.2	3.6	75.8	20.9	3.3	81.0	16.0	3.0	92.4	6.2	1.4
BHDB‡	24.9	57.2	17.9	80.7	10.2	7.1	93.2	3.8	3.0	—	—	—	—	—	—

* 10% of the radioactivity in the presence of $HgCl_2$ ran near the front. This was presumably iodide (10).

† Ethylene diamine tetraacetic acid.

‡ n-butyl parahydroxy 3,5 diiodobenzoate.

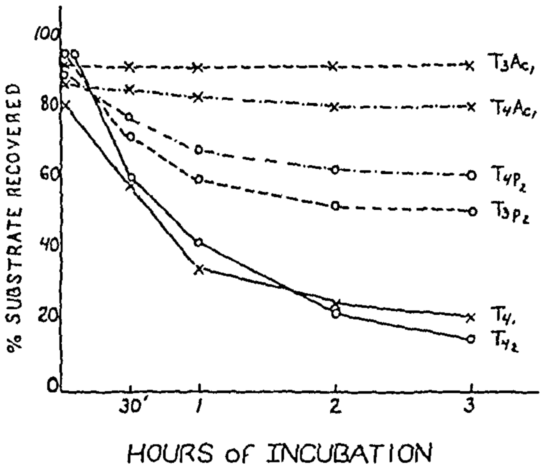

FIG. 1. The deiodination of the acetic and propionic acid analogues of thyroxine and triiodothyronine (10^{-8} *M*) by rat liver slices. The same subscript numbers indicate the same experiment. A different experiment (Table IV) indicated that there was slight deiodination of the acetic acid analogues.

nine studied to be deiodinated at least as easily and rapidly as the tetraiodo form.

Inhibition of deiodination by other iodinated thyronines and the acid analogues

Table 5 indicates that the deiodination of radiothyroxine and radiotriiodothyronine was inhibited by 10^{-4} *M* carrier thyroxine, triiodothyronine, and their acetic and propionic acid analogues. The deiodination of thyroxine was inhibited, in order of decreasing magnitude, by thyroxine, triiodothyronine, propionic acid analogues and acetic acid analogues. Triiodothyronine was inhibited about equally by all the iodinated thyronines tested though possibly less by the acetic and propionic acid analogues of thyroxine.

TABLE 4. DEGRADATION OF L-THYROXINE, TETRAIODOTHYROPROPIONIC AND ACETIC ACIDS, AND TRIIODOTHYROPROPIONIC AND ACETIC ACIDS BY RAT LIVER AND MUSCLE HOMOGENATE

	% total thyroxine 10^{-8}M distributed as			% total thyroxine 10^{-8}M distributed as		
	T4	I^-	O	T4	I^-	O
Control	97.9	2.1	0	80.6	16.6	2.8
Liver	47.4	42.9	9.7	—	—	—
Muscle	31.5	65.5	3.0	10.8	80.2	8.9
	% total tetraiodothyropropionic acid 10^{-8}M distributed as			% total tetraiodothyroacetic acid 10^{-8}M distributed as		
	T3PA	I^-	O	T4AC	I^-	O
Control	94.9	5.1	0	92.8	7.1	±
Liver	89.1	9.9	1	—	—	—
Muscle	83.8	14.7	1.5	83.4	13.5	3.2
	% total triiodothyropropionic acid 10^{-8}M distributed as			% total triiodothyroacetic acid 10^{-8}M distributed as		
	T4PA	I^-	O	T3AC	I^-	O
Control	91.2	8.3	0.5	95.4	4.6	0
Liver	82.3	15.9	1.8	—	—	—
Muscle	80.3	17.2	2.5	85.0	14.0	1.0

TABLE 5. DEGRADATION OF L-THYROXINE AND L-3,5,3′-TRIIODOTHYRONINE BY RAT LIVER SLICES IN THE PRESENCE OF VARIOUS IODOTHYRONINES

Substance added	Conc.	L-thyroxine 4.7×10^{-7}M			L-triiodothyronine 2.6×10^{-7}M		
		% of total distributed as			% of total distributed as		
		T4	I	O	T3	I	O
None		28.5	57.9	13.6	75.1	18.8	6.1
Triiodothyronine	3.2×10^{-4}	66.9	19.8	13.2	95.5	3.0	1.5
Thyroxine	2.0×10^{-4}	85.5	6.5	8.0	94.5	3.5	2.0
Triiodothyroacetic acid	3.1×10^{-4}	53.6	31.0	15.5	94.2	3.6	2.2
Tetraiodothyroacetic acid	3.0×10^{-4}	42.2	37.1	20.7	85.4	8.6	6.0
Triiodothyropropionic acid	3.3×10^{-4}	64.0	25.0	11.0	94.5	4.5	1.0
Tetraiodothyropropionic acid	3.4×10^{-4}	61.8	22.8	15.4	87.9	7.4	4.7

Deiodination of endogenously labelled thyroxine

The incubation of endogenously labelled thyroxine with rat liver slices resulted in the formation of radioiodide. No material with the R_f of 3,5 diiodothyronine or any other new compound was identified chromatographically. A small amount of a compound could have gone undetected because there was little radioactivity, but in the five solvent systems used no new material of a significant amount, relative to iodide and thyroxine, was noted.

DISCUSSION

The liver, kidney and muscle (but not the thyroid (10, 11)) of rats deiodinated radiothyroxine and radiotriiodothyronine and, to a certain extent, their acetic and propionic acid analogues. This system had an optimal temperature (although the present study confirms the relative heat stability of the homogenate (12)), required oxygen, was inhibited by boiling, certain toxic compounds (some heavy metals, phenols, quinone, cyanide) estrogenic substances and BHDB. It was strikingly unaffected by many antithyroid compounds, other iodinated compounds and a sulphydryl inhibitor (p-chloromercuribenzoate).

The present finding of inhibition of deiodination of thyroxine by estrogenic substances is in contrast to that of Cruchaud *et al.* (13). These authors, however, used estradiol monophosphate in a smaller concentration. Such an inhibition might be an additional cause for the rise in blood levels of thyroxine in humans with high estrogen levels. *In vivo* human studies, as yet incomplete, suggest, in several instances, the possibility of a block by stilbesterol of the deiodination of thyroxine.

The main product of this deiodinating system was radioiodide. The absence of radiotriiodothyronine, in spite of its being less rapidly deiodinated than thyroxine, suggests that it was not formed. The same reasoning applies to the acetic and propionic acid analogues. The substrates were labelled only in the prime positions and therefore 3,5 diiodothyronine or a derivative thereof, would have gone undetected. No 3,5 diiodothyronine was found, however, in the deiodination of endogenously labelled thyroxine but, since we do not know how rapidly 3,5 diiodothyronine may be deiodinated, we cannot conclude that it was not formed. The finding that 3,5 diiodothyronine did not inhibit the deiodination of thyroxine suggests that it was not or, if so, only slightly deiodinated by the same enzyme system. In addition, 3,5 diiodothyronine should be more difficult to deiodinate than thyroxine. Of the two possibilities, 3,5 diiodothyronine was not formed during the deiodination of thyroxine or 3,5 diiodothyronine was formed but was rapidly metabolized, the former would, therefore, seem the more likely. As previously noted, the radioactivity was small and the possibility of 3,5 diiodothyronine derivatives or other compounds being formed in small quantities cannot be ruled out. These results suggest that either a compound was formed with the chromatographic characteristics, in five different solvent systems, of thyroxine or iodide, or thyroxine was deiodinated, at least to a major extent, to iodide and thyronine (or some completely deiodinated thyronine derivative).

Others have found an accumulation of radiotriiodothyronine (1, 2, 3 13), acetic acid analogues (3, 5, 6) propionic acid analogues (3), pyruvic acid analogues (7, 8) and glucuronide acid conjugate (9) from radiothyroxine metabolized either *in vivo* or *in vitro*. In the present study deiodination by homogenates was resistant to boiling compared to deiodination by slices or mitochondria and BHDB inhibited deiodination by slices much more than deiodination by mitochondria. There may, therefore, be several enzyme systems (deiodinating, deaminating, decarboxylating) capable of metabolizing thyroxine and the results may vary with the tissue studied and the method of study.

The question of where the deiodinating enzyme system is remains unresolved. Mitochondria seemed particularly effective in the system studied here. A deiodinating system at the cell surface, however, might serve to deiodinate thyroxine to facilitate its entrance into the cell and might explain why thyroxine is more rapidly deiodinated than triiodothyronine. There was no evidence in the present study, however, for the formation of any iodinated thyronine by the deiodination of thyroxine; nor was there any evidence to indicate whether the deiodinating system acted within or on the surface of the cell in the intact tissue. Furthermore, deiodination may have no relation, directly, to the metabolic activity of the thyroid hormone, in spite of the fact that BHDB both reduces oxygen consumption and inhibits deiodination (12).

The deiodinating system presently under consideration seems specific principally for tetra and triiodinated thyronine compounds. The addition of tetra- and tri-iodinated thyronines inhibited the deiodination of thyroxine or triiodothyronine to an extent often commensurate with the amount of deiodination which these compounds underwent when added alone to a similar system. 3,5 Diiodothyronine did not inhibit deiodination of thyroxine, nor did the iodinated tyrosines, iodide, Priodax, Telepaque or Teridax. It is of interest, however, that an iodinated single benzene-ring compound (BHDB) did inhibit deiodination suggesting that it may be deiodinated itself, and indicating a lack of discrete specificity for the enzyme system involved.

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THE METABOLISM OF I^{131}-LABELED THYROXINE, TRIIODOTHYRONINE AND DIIODOTYROSINE BY AN ISOLATED PERFUSED RABBIT LIVER[1]

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ABSTRACT

Isolated rabbit livers were perfused for 12 to 15 hours with I^{131}-labeled thyroxine, triiodothyronine and diiodotyrosine in order to evaluate the hepatic metabolism of these substances. The livers were able to concentrate these amino acids in appreciable quantities showing somewhat greater accumulation in livers perfused with labeled triiodothyronine than with thyroxine. Triiodothyronine and thyroxine were metabolized by the perfused liver at rates identical in ratio to the intact rabbit but faster by a factor of almost six. Diiodotyrosine, however, was deiodinated at a rate similar to that in the intact animal. Measurements of biliary excretion of radioactivity showed that this pathway proved more important over the period studied for livers perfused with triiodothyronine than for those perfused with thyroxine. Twenty-six per cent of initial radioactivity was excreted over 12 hours in the bile of the former and 8% in the latter. Less than 3% of the initial perfusate radioactivity appeared in the bile and over 12 hours when perfusion with labeled diiodotyrosine was carried out.

The perfused liver proved capable of producing triiodothyronine by the deiodination of thyroxine in the majority of studies. Several unidentified compounds appeared in both the bile and the plasma of the perfusate following perfusion with both thyroxine and triiodothyronine. It is likely that the most abundant of these components is a glucuronide conjugate of thyroxine and triiodothyronine.

THE metabolism of thyroid hormone by the liver has been a matter of considerable interest since 1919 when Kendall (1) showed that circulating thyroxine is partly collected by the liver and that derivative iodides can be recovered in the bile. Using radioisotopic and chromatographic techniques, many recent investigators have restudied this subject. Information, however, is still lacking as to the precise role played by the liver in the peripheral metabolism of thyroid hormones.

The importance of the enterohepatic circulation in the metabolism of thyroxine has been emphasized by Albert and Keating (2) and by Myant (3). There is considerable evidence that the liver plays a key role in the regulation of the thyroid hormone content of the body by destruction of excess circulating hormone and regulation of biliary excretion mechanisms. Several workers have investigated the nature of thyroid hormone derivatives in bile, and the importance of the glucuronide conjugation mechanism in the metabolism of thyroxine has been well established (4, 5, 6, 7, 8). In addition to glucuronides, recent work (9) has shown that the iodothyropyruvic acid derivatives of both thyroxine and triiodothyronine may be found in the bile of rats.

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[1] This work was carried out in part at the Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas. Presented at the 1958 Meeting of the American Goiter Association.

The site of origin of triiodothyronine in the plasma has not been established. There is appreciable evidence that it may arise from the deiodination of thyroxine (7, 10, 11, 12, 13, 14) although agreement is not unanimous (15). *In vitro* studies (16, 17, 18, 19, 20) indicate that the liver may be one of the sites of peripheral deiodination.

Using a perfusion apparatus, it was felt that it might be possible to isolate the action of the liver in the metabolism of thyroid hormones and to investigate its function, not only as part of an excretion mechanism, but for its possible importance in the deiodination of these substances.

METHODS

The perfusion technique used in these studies was described previously by Young, Prudden and Stirman (21). In this procedure, the liver of an anesthetized adult New Zealand rabbit was carefully removed following cannulation of the portal vein, inferior vena cava, and the bile duct with polyethylene catheters. The liver, which weighed about 100 grams, was placed in the apparatus with the bile duct cannula emptying into a collection tube. The perfusate was a 50–50 mixture of defibrinated, heparinized rabbit blood and Hartman's solution with a total volume of 1000 cc. It was oxygenated by permitting it to flow freely from the vena cava through a Vigreaux fractionating column connected to an oxygen source. After collection in a mixing chamber the perfusate was then pumped to a hydrostatic reservoir from which it flowed into the liver through the portal vein at a rate of about 150 cc. per minute. The apparatus was kept in a thermostatically controlled box at a temperature of 37° C. In order to avoid bacterial contamination, the entire apparatus was cleaned and autoclaved, and sterile technique was used in the preparation of the perfusate. In addition, Aureomycin was added to the perfusate at four hour intervals during the procedure. The average liver was perfused for a period of 12 to 15 hours.

Commercially prepared I^{131}-labeled *l*-thyroxine, 1-3,5,3′-triiodothyronine and diiodotyrosine,[2] in amounts of 1 to 10 micrograms containing from 30 to 150 microcuries of I^{131} were used as the substrate. This material as received showed various degrees of impurity and was purified by paper chromatography before use. In several of the procedures, the starting thyroxine was contaminated with a small amount of triiodothyronine and iodide despite purification. The labeled amino acid was thoroughly mixed with the perfusate which was then added to the apparatus. A control sample of perfusate was taken before the liver was placed in the apparatus, a second sample taken immediately after the start of perfusion of the liver and a third 5 minutes later. Additional samples were taken every 30 minutes for the first two hours and hourly thereafter.

The radioactivity of the plasma of the perfusate and of the bile was determined by measurements in a scintillation well counter. The proteins of the identical plasma aliquot were precipitated using 5 volumes of 20% trichloracetic acid. The precipitate was washed twice with 5% trichloracetic acid and then measured in the well counter to determine the protein-bound I^{131} (PBI^{131}).

Measurements of total protein, albumin (22) and urea nitrogen (23) of the perfusate were made every two hours. Samples of the perfusate for chromatography were obtained before the liver was added to the apparatus and every three hours thereafter. When bile flow permitted, samples taken at similar intervals were used in the examination of the bile.

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[2] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

Samples of the plasma of the perfusate and the bile were prepared for chromatography by acidifying with 0.2 cc. of 5N HCl per cc. of sample and then extracting twice with 5 cc. of *n*-butanol (24). The supernatants were combined and reduced to dryness by vacuum distillation at room temperature. The residual was dissolved in a small amount of butanol and chromatographed in an ascending butanol-dioxane-ammonia system (25). The dioxane was purified by distillation. Selected samples were chromatographed, in addition, in an ascending butanol-acetic acid system (26). Appropriate carriers were applied to all chromatograms before development and were identified with ninhydrin or sulfanilic acid (27). All chromatograms were radioautographed on no-screen X-ray film and then scanned with an automatic geiger counter and recording rate-meter. Radioautographic spots were identified by comparison of the film with the location and shape of the carrier spot on the chromatogram. The radioactivity on the chromatogram was quantitated by planimetry of the rate-meter recording after identification and delineation of each peak by comparison with the corresponding radioautograph.

RESULTS

Seven technically satisfactory perfusions with thyroxine, four with triiodothyronine and one with diiodotyrosine were completed. Over the course of the average procedure the pH of the perfusate remained relatively constant. Measurement of albumin and globulin showed little change. The urea nitrogen accumulated according to a single exponential cumulative equation and doubled by the end of 12 hours. This pattern of urea increase was taken to indicate the presence of actively functioning hepatic tissue. The mean rate constant of the expression for urea accumulation for those livers perfused with thyroxine was 0.00181 min.^{-1}. This represents a half-life of accumulation of 383 minutes. For those livers perfused with triiodothyronine, the same formulation showed a mean rate constant of 0.00118 min.^{-1} (half-life = 587 min). When the difference between these mean values was examined by "t" test, it was found, interestingly enough, that the difference was significant ($P = 0.01$). Hence urea nitrogen accumulated significantly more rapidly in those livers perfused with thyroxine compared with those perfused with triiodothyronine. The physiological implication of this finding is not immediately apparent.

Control procedures included circulation of the perfusate with its labeled compound for twelve hours in the apparatus without a liver. In these control studies, the total radioactivity, protein-bound I^{131} and chromatograms all showed no significant change from the starting determinations. During the course of those control studies without the liver, measurement of the perfusate urea nitrogen (which was essentially constant) showed a standard error of 1.5% of the mean perfusate value. Similarly, measurement of the PBI^{131} showed a standard error of the hourly measurement of 0.27% of the mean. The measurement of total plasma radioactivity showed a standard error of 0.83%. These figures represent the intrinsic errors of the procedures and are considered satisfactory. Perfusion of sodium radioiodide through a liver for the same period showed no changes in the indices studied. The iodide was excreted rapidly in the bile. Over the course of the control experiments there was a loss of radioactivity (presumably adsorbed to glassware) equivalent to 0.75% of the total activity per hour.

RATE STUDIES

Perfusate

The total I^{131} of the perfusate showed a rapid decrease over the first two hours. At the end of two hours the livers perfused with thyroxine had accumulated a mean of 24% while those perfused with triiodothyronine had accumulated a mean of 39% of total radioactivity. This difference was statistically significant ("t" test, P·0.01). The total perfusate activity in all studies then remained at a fairly constant level for the duration of the procedure. Since the system is a closed one, this first phase of disappearance of radioactivity was interpreted as indicating either passive or active distribution of radioactivity within the liver.

Measurements of the PBI^{131} of the plasma of the perfusate showed that triiodothyronine was deiodinated at a significantly faster rate than was thyroxine (Fig. 1). The mean half-life for the disappearance of protein-bound I^{131} when the liver was perfused with thyroxine was 32.5 hours while that for triiodothyronine was 22.2 hours. This difference is significant. The single satisfactory procedure in which the liver was perfused with diiodotyrosine showed an appreciably different and much faster rate of deiodination (half-life, 9.4 hours).

By contrast, measurements of the relative radioactivity of each peak appearing on the chromatograms permitted the estimation of the disap-

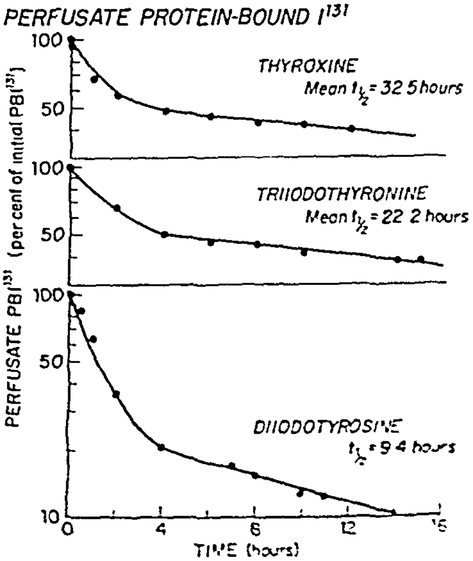

FIG. 1. The disappearance of the perfusate protein-bound I^{131} for livers perfused with I^{131}-labeled thyroxine, triiothyronine and diiodotyrosine. The half-lives for thyroxine and triiodothyronine are the means of 7 and 4 experiments respectively, while that for diiodotyrosine represents a single study. The ordinate represents the logarithm of the perfusate PBI^{131} while the abscissa represents time in hours.

pearance rates for chromatographically pure thyroxine and triiodothyronine. Here, the thyroxine disappeared from the perfusate with a mean half-life of 24.3 hours while triiodothyronine was removed at a significantly faster rate with a mean half-life of 9.5 hours. The disappearance rates, calculated in this fashion, were thus somewhat faster than when calculated from the determinations of the perfusate protein-bound I^{131} (see above).

Analysis of the perfusion data for the kinetic rate constants shows that the specific rate of liver degradation for thyroxine or triiodothyronine is directly related to the weight of the liver. The rate constant "k" (% degraded per hour per 100 gm. of liver) is 3.8% for thyroxine and 8.0% for triiodothyronine. Further calculation indicates an average degradation of 1 to 2 μg. of thyroxine per 100 gm. of liver for a twelve hour period.[3]

Bile

Over a period of twelve hours, those livers perfused with thyroxine excreted in the bile a mean of 8% of the total radioactivity initially added to the perfusate. However, radioactivity derived from triiodothyronine was found to have a total biliary excretion over the same period of a mean of 26% of total radioactivity. Thus despite equality of bile production (averaging 2 to 6 cc. an hour) with both amino acids, the perfused liver excreted in the bile three times as much radioactivity derived from triiodothyronine as compared to that derived from thyroxine. The biliary excretion curves of radioactivity for thyroxine perfused livers showed an early peak of radioactivity (three to four hours) followed by a steady fall. Following labeled triiodothyronine administration however, radioactivity was excreted in the bile with a different pattern, showing a slow rise in hourly excretion reaching a peak by 7 to 8 hours and then falling off slightly.

In the liver perfused with labeled diiodotyrosine, a total of only 2.4% of the total perfusate radioactivity appeared in the bile over a 12 hour period.

CHROMATOGRAPHIC STUDIES

Perfusion with I^{131}-labeled thyroxine

In those livers perfused with labeled thyroxine, chromatographic studies of the perfusate showed that thyroxine gradually decreased with time while iodide, several unknown materials and triiodothyronine appeared (Fig. 2). Similarly, chromatograms of the bile showed all of these compounds (Fig. 3).

The relative quantities of radioactivity in each compound separated on the chromatograms of the perfusate for a typical liver perfused with I^{131}-labeled thyroxine are shown in Figure 4. Thyroxine decreased in a moderately slow and irregular fashion with a drop over 12 hours to a mean of 48% of initial radioactivity. Triiodothyronine appeared in the perfusate

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[3] The kinetic formulation used in these calculations will be presented in a subsequent publication.

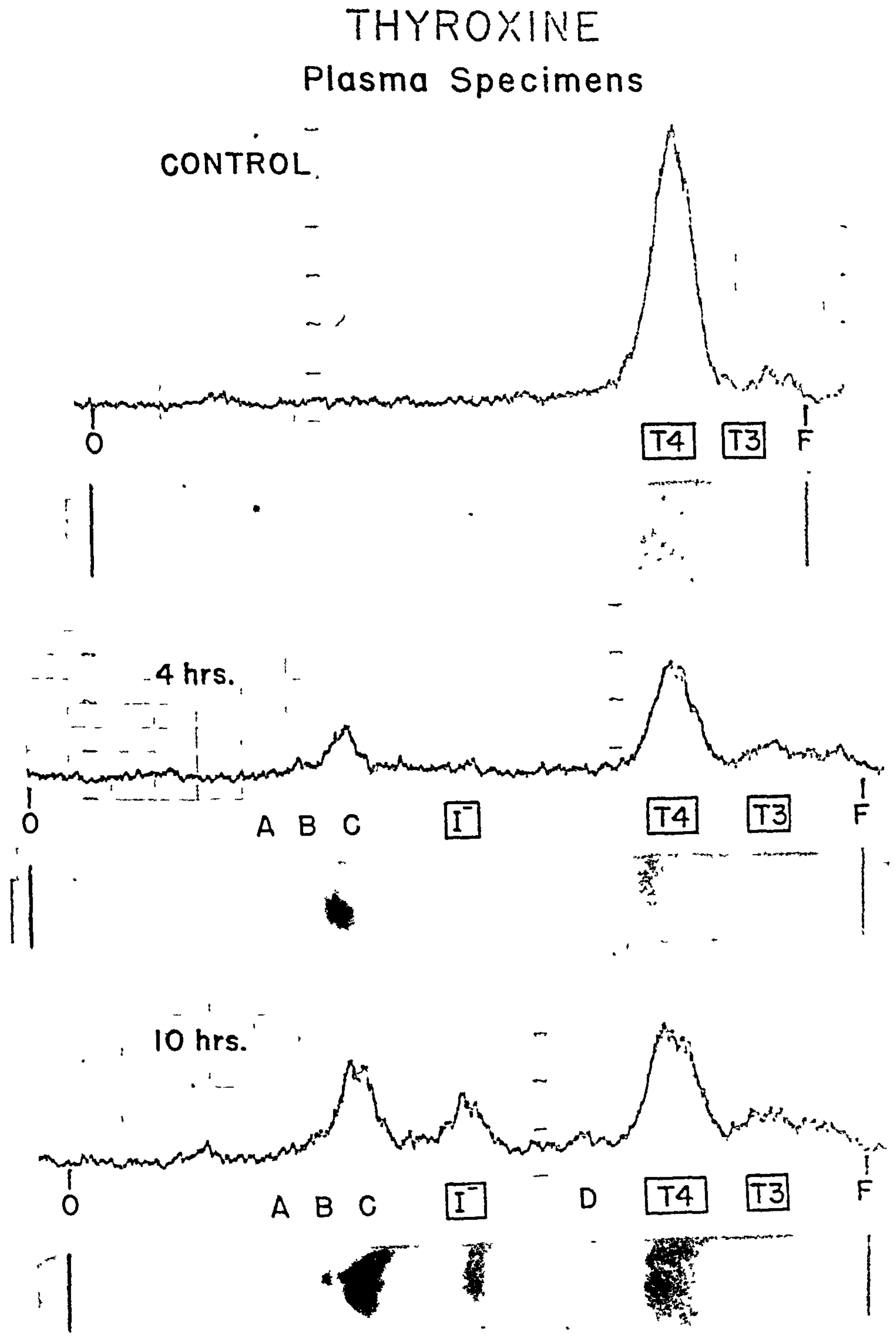

FIG. 2. Perfusion of a liver with I^{131}-labeled thyroxine. The radioautographs of chromatograms of the plasma of the perfusate are placed beneath the corresponding ratemeter recordings of radioactivity content. Legend: O = origin, F = front, T4 = thyroxine, T3 = triiodothyronine, I^- = iodide. The unknown peaks are labeled A, B, C, and D.

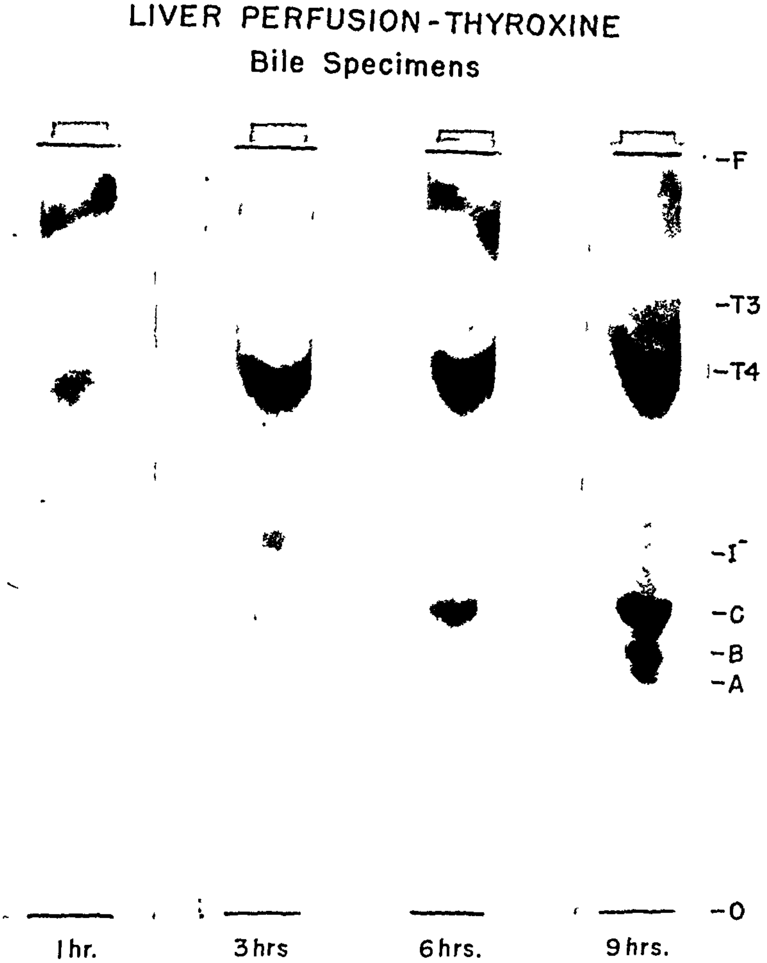

Fig. 3. Perfusion of a liver with I^{131}-labeled thyroxine. Radioautographs of bile samples obtained 1, 3, 6, and 9 hours after the start of perfusion. The legend is the same as in *Fig.* 2.

in significantly increased quantities in four of the seven procedures in which the liver was perfused with thyroxine. The remaining three started with appreciable quantities of triiodothyronine and the area under this peak remained relatively constant throughout. The main product of the breakdown of thyroxine was iodide which slowly and steadily increased to reach its maximum concentration of 8 to 25% (mean 15%) of total chromatogram activity by 12 hours (Table 1). Several unknown compounds appeared and the predominant one, Unknown C (probably a glucuronide as indicated below) reached a mean value by the end of 12 hours of 7.2% of total chromatogram activity. Two other unknowns, A and B, appeared less constantly and in smaller quantities. Unknown B usually reached its maximum concentration of 2 to 3% by the sixth to the ninth hour and then decreased. Unknown A was found in the plasma on two occasions, and Unknown D was seen only once.

Analysis of the chromatograms of the bile for a typical liver perfused

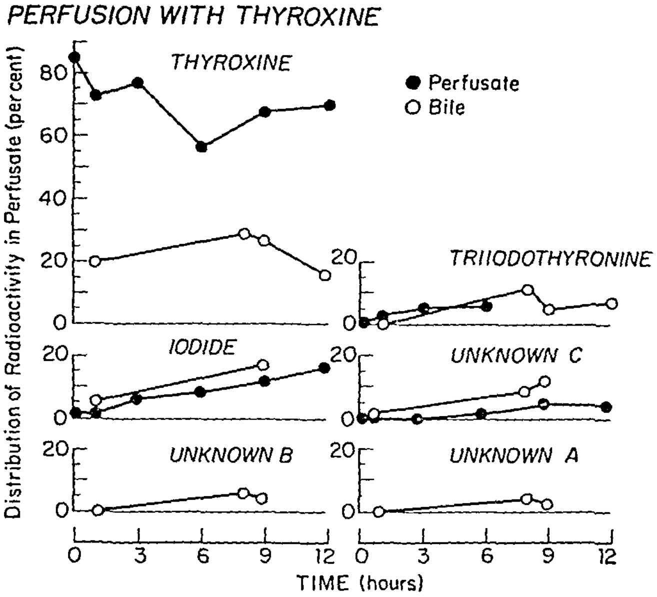

FIG. 4. Distribution of the relative radioactivity content of chromatograms of the perfusate (solid circles) and bile (open circles) of a liver perfused with I^{131}-labeled thyroxine. The ordinate represents the percent of the total chromatogram radioactivity while the abscissa represents the time of the samples studied.

with labeled thyroxine (Fig. 4) showed that thyroxine was the major component as early as thirty minutes after perfusion was started at which time the first bile specimen was examined. The thyroxine content of bile over the period of twelve hours remained relatively constant. Triiodothyronine and iodide appeared in all bile specimens in about the same concentration that was seen in the plasma (in terms of total activity of the chromatogram). Unknown C not only appeared in the bile much earlier than in the perfusate, but was usually present there in much greater concentration (Table 1). At the end of 12 hours Unknowns A and B were found in the bile in all thyroxine perfusions with respective mean concentrations of 4.3% and 9.6% of total chromatogram activity.

TABLE 1. DISTRIBUTION OF CHROMATOGRAM RADIOACTIVITY

(All values are means; they are expressed as per cent of the total chromatogram radioactivity upon termination of perfusion.)

Perfusate	Specimen	Thyroxine	Triiodothyronine	Iodide	A	B	C	D
I^{131}-thyroxine	perfusate	48	5.8	15	—	—	7.2	
	bile	24	5.9	16	4.3	9.6	19	
I^{131}-triiodothyronine	perfusate	4.4*	21	14	2.7	11	22	7.0
	bile	7.0	15	11	3.6	4.9	38	8.8

* Contaminant of the triiodothyronine initially added to perfusate.

Perfusion with I^{131}-labeled triiodothyronine

Chromatographic studies of the perfusate of those livers perfused with labeled triiodothyronine showed qualitatively similar results to those described above for the thyroxine. Similar measurements of the relative content of the substances found in the perfusate and the bile are shown during the course of a typical perfusion in Figure 5. In the perfusate, chromatography of the control specimens showed that a mean of 83% of the initial total perfusate radioactivity was present as triiodothyronine. After twelve hours, however, the amount of triiodothyronine had dropped sharply to 21% of initial radioactivity. In almost all procedures where I^{131}-triiodothyronine was added to the perfusate, thyroxine in quantities of 5 to 10% was present as an initial contaminant. Unknown C with a mean perfusate concentration of 22% by the end of 12 hours was present in much greater concentration than was seen when thyroxine was the starting material. Iodide made up about 14% of the total radioactivity and Unknown B was present in rather high concentrations (mean 11%). Unknown A was found in all plasma samples with a mean concentration at the termination of the perfusion of 2.7% of the total chromatogram activity (Table 1).

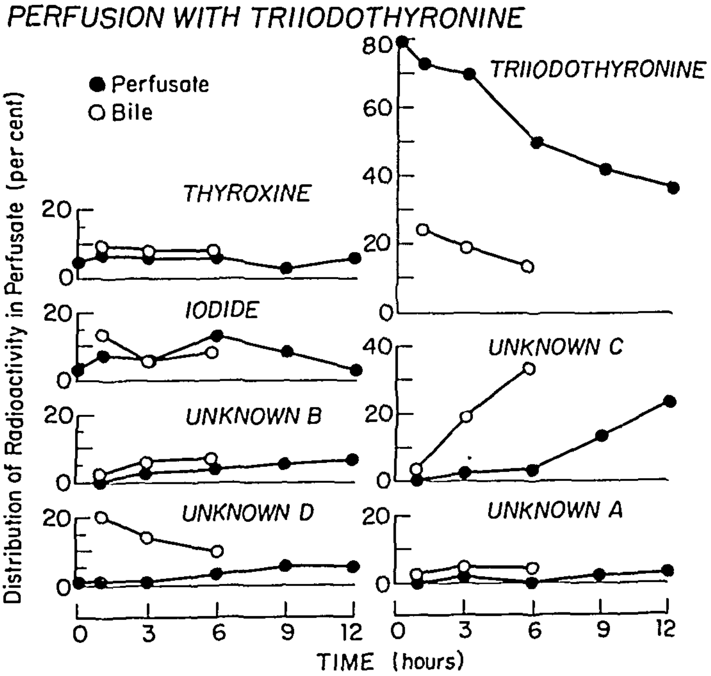

FIG. 5. Distribution of the relative radioactivity content of chromatograms of the perfusate and bile of a liver perfused with I^{131}-labeled triiodothyronine. The ordinate and abscissa are identical with that in Fig. 4.

Examination of the bile showed that the major component was Unknown C. This compound, in those livers perfused with triiodothyronine, contributed in 12 hours more than one third of the total biliary activity; an amount twice as great as that found following perfusion with thyroxine.

Unknowns A and B were present in all late bile samples but in relatively small quantities. Unknown D was found in all specimens of bile and was the only derivative compound that appeared initially (one hour) in higher concentrations (16%) than was seen after twelve hours of perfusion.

Hydrolysis with β-glucuronidase of Unknown C eluted from the chromatogram yielded a substance that traveled on the chromatogram to the same location as carrier triiodothyronine.

Perfusion with I^{131}-labeled diiodotyrosine

In those livers perfused with labeled diiodotyrosine, chromatograms showed the rapid disappearance of diiodotyrosine from the perfusate concomitantly with the appearance of iodide. Before perfusion started, 97% of the total radioactivity of the chromatogram was present as diiodotyrosine. However, iodide appeared rapidly in the perfusate with a concentration by the end of the 2nd hour of 23%, by the end of the 6th hour of 40% and by the end of the 12th hour of 46% of chromatogram radioactivity. Examination of the bile showed the early presence of both diiodotyrosine and iodide but as perfusion continued, the former decreased to minimal quantities while the latter accounted for almost all of the radioactivity of the chromatogram.

DISCUSSION

The viability of the liver in the perfusion preparation is reasonably assured by the findings of the rising urea nitrogen of the perfusate, maintenance of total protein levels, and continued production of bile in appreciable quantities. Microscopic examination of the liver upon termination of each study showed intact morphology. Only those perfusion studies that met the above criteria were considered to be satisfactory and included in the series.

It is interesting to note that the relative rates of deiodination of thyroxine and triiodothyronine by an isolated perfused liver are roughly analogous to those obtained in intact animals where triiodothyronine is not only removed from the blood more rapidly than thyroxine but is deiodinated to a greater degree (28, 29, 30). *In vivo* studies in rabbits of the same breed used in these perfusion experiments showed that the average half-time of disappearance of plasma radioactivity after administration of labeled thyroxine was 192 hours, that for triiodothyronine 125 hours and that for diiodotyrosine 14.5 hours. (31) The disappearance rates of the protein-bound I^{131} obtained by liver perfusion (32.5 and 22.2 hours respectively) are faster by a factor of almost 6 than those seen for both thyroxine and triiodothyronine in the intact animal. It is striking to note that the ratio of the degradation half-time of thyroxine compared with that for triiodothyronine is virtually identical for both the perfusion preparation and the intact animal. However, for the liver perfused with diiodotyrosine, the deiodination rate is very similar to that in the intact animal (9.4 and 14.5

hours, respectively). This is of interest in view of Flock's (7) observation that the rate of deiodination of diiodotyrosine was not altered by evisceration of the rat. The rate of deiodination of both thyroxine and triiodothyronine was reduced in the eviscerated rat over that seen in the intact animal although triiodothyronine continued to disappear from the blood more rapidly than thyroxine. *In vitro* studies (32) indicate that the liver readily metabolizes diiodotyrosine; however Flock's observations and those of the present study suggest that the liver may not play a major role in the deiodination of diiodotyrosine in the intact animal.

In the present study, a greater role by a factor of three was played by the bile of those livers perfused with triiodothyronine than in those perfused with thyroxine in terms of the amount of total radioactivity excreted. Cooper and Beringer (33) noted no significant difference in the biliary excretion of radioactivity following administration of labeled thyroxine and triiodothyronine to bile fistula dogs, finding one-third of the administered dose in the bile by 120 hours. Roche, however, noted that 60% of radioactivity derived from injected I^{131}-labeled triiodothyronine appeared in the bile of rats by 15 hours compared to 40% of that derived from I^{131}-labeled thyroxine.[4]

The chromatographic findings of triiodothyronine in the perfusate and the bile of livers perfused with labeled thyroxine suggest the ability of the liver to deiodinate thyroxine. *In vitro* studies by several workers have shown that liver tissues have the capability of deiodinating thyroxine (16, 17, 18, 19, 20) and the existence of deiodinase systems in the liver has been demonstrated by others (34, 35). In addition, Flock (8) has shown that the hepatectomized dog does not deiodinate thyroxine and triiodothyronine as completely as the normal animal. The present findings therefore add further evidence for the existence of a peripheral mechanism for the formation of triiodothyronine from thyroxine and suggest that the liver may play an important role in this function.

Of the unknown compounds noted in the perfusate and bile in the present experiments, there is suggestive evidence that the major one (Unknown C) is a glucuronide. Briggs *et al.* (4) in a study in which a rat liver was perfused with labeled thyroxine found in the bile appreciable quantities of a substance subsequently identified as the glucuronide conjugate of thyroxine (5). Roche *et al.* (9) and Flock and Bollman (7) identified this substance in bile.

The presence of thyroxine glucuronides in the plasma has been described only in animals whose bile ducts have been ligated (37) or whose livers have been severely damaged (20). In the present studies a compound that was probably a glucuronide was found in appreciable quantitites in the perfusate following perfusion of livers with thyroxine and triiodothyronine. Its presence may indicate undetected biliary damage. More likely, however, since the perfusion takes place in a closed system, it is the result of the absence of a urinary excretion mechanism which could handle the rapid removal of this material from the plasma.

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[4] Data presented by Roche during the discussion (p. 224) of a paper by Vannotti (20).

Acknowledgment

The authors are deeply indebted to Mr. Leon Woods and Mr. Kenneth G. DuBois for their skillful technical assistance. The help of Mr. Eladio Nunez and Mr. Nathanial Griffin is also gratefully acknowledged. Dr. Melvin Schwartz was kind enough to aid in the analysis of the data.

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RELATION OF NUCLEIC ACIDS TO THE STRUCTURE AND FUNCTION OF THE GUINEA PIG THYROID GLAND

JOSIP MATOVINOVIC AND AUSTIN L. VICKERY

ABSTRACT

Groups of guinea pigs were administered thyrotropin, thyroxine, and thiouracil. The weight, functional activity (I^{131} uptake), relative percentages of tissue constituents, average cell height and the cell population density of the thyroid glands were determined. Desoxyribonucleic acid and ribonucleic acid of thyroid tissue were determined by chemical methods. Since the histological structure of the thyroid gland is a variable factor, it should be considered in the interpretation of data obtained by chemical analysis of whole tissue samples. A method is described for expressing the results of chemical analyses of thyroid gland nucleic acids in terms of a calculated thyroid parenchymal cell mass. These results differ in significance from the conventionally expressed values based on a unit weight of whole tissue sample. The nuclear content of DNA was found to be independent from the stimulating action of thyrotropin. In the thyrotropin stimulated gland, an increase of RNA was observed. In the absence of thyrotropin stimulation, a decrease in RNA occurred. Under the stimulation of thyrotropin, the increase in total RNA was proportionally greater than the increase in average weight of the gland. The increase of total RNA per gland was less marked when related to the increase in total cell mass of the gland. In the absence of thyrotropin stimulation (thyroxine-injected animals), the decrease in total RNA per gland was proportionally greater than the decrease in average weight of the gland; but this effect was more pronounced when the decrease in total RNA was related to the decrease in total cell mass of the gland. Thiouracil *per se* probably does not inhibit the synthesis of RNA in the thyroid cell.

THERE is an abundant literature on the nucleic acids of most mammalian organs but relatively little concerning the thyroid gland. Nucleic acids of the thyroid have been determined by histochemical (1, 2), histospectroscopic (3, 4), and chemical methods (5, 6, 7, 8). The results of histospectroscopic studies are expressed in arbitrary units, while the data of chemical analyses are usually reported as concentration per unit of wet weight of tissue or content per whole gland.

The structure of the thyroid gland makes it difficult to interpret chemical assays if expressed in terms of the weight of whole tissue. Since the thyroid is not a solid organ, but contains follicular spaces which constitute a large and variable factor, it might prove helpful to express the results of chemical analyses in terms of a calculated cell mass which excludes stromal elements and colloid. This paper is concerned with an histological and chemical technique which has provided a measurement of the nucleic acids per unit weight of thyroid protoplasm. The method has been utilized in studies of the effects of thyrotropin, thyroxine, and thiouracil upon the metabolism of nucleic acids in the guinea pig thyroid gland.

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METHODS

Male guinea pigs weighing 165–210 gm. were used in these experiments. Each group consisted of five animals fed *ad libitum* with a regular diet and lettuce. Control and experimental groups of animals were sacrificed after 14 days and after 3 months. One group received 0.5 U.S.P. units of thyrotropin (Armour) in 0.5 ml. saline subcutaneously daily for 14 days. One group received 100 μg. of *l*-sodium thyroxine in 0.5 ml. saline (pH 8.5) daily subcutaneously for 14 days. One group was fed with 0.2% thiouracil ground in food for 3 months. The weight of all animals was regularly recorded. Animals were sacrificed by a blow on the head and exsanguination. Eight hours before being sacrificed they were injected with 0.5 μc. I^{131} in 0.5 ml. saline subcutaneously. The food was not omitted until the end of the experiment. The I^{131} in each thyroid gland was counted in a well-type scintillation detector, and counts were expressed as per cent of administered dose. Aliquots of thyroid tissue of thiouracil-fed animals were also precipitated with 20% trichloracetic acid and then counted in a well-type scintillation detector. Homogenized aliquots of these thyroids were digested with trypsin and chymotrypsin and thereafter were chromatographed in a butanol-2 N acetic acid solvent system. Strips were counted in segments in a well type scintillation detector. The thyrotropin and thyroxine-treated animals gained weight during 7 days of the control period. In the experimental period, despite increased food intake, the thyrotropin and thyroxine-treated animals lost $\frac{1}{6}$ and $\frac{2}{3}$ respectively of the weight which they had gained during the control period. Both groups, especially the thyroxine-treated animals, had evidence of hypermetabolism. Control groups and thiouracil-fed animals gained weight throughout the experiment. The thiouracil-fed animals did not develop signs of hypometabolism.

Histological Methods

A calculated cell mass value may be obtained by an histometrical determination of the relative percentages of thyroid tissue constituents. One medial part of each guinea pig thyroid lobe was fixed in 10% formalin, routinely processed and embedded in paraffin. Sections of 6μ thickness were cut from the median portion and stained by hematoxylin and eosin, Feulgen, and Masson tri-chrome techniques. Quantitative estimates of the relative percentages of thyroid tissue components were made by the method of Chalkley (9). This consists of counting a series of random tissue contacts in a number of high power microscopic fields made by points fixed in the microscope eye-piece. A ratio is thus obtained by comparing the total number of random ocular point intersections for each tissue constituent. Parenchymal thyroid cells, follicle spaces, and stroma were the tissue fractions studied. Homogeneity of tissue architecture is desirable for a reasonably accurate application of this sampling technique. The thyroids were quite uniform in structure, as was demonstrated by obtaining similar results on tissue composition from sections cut at three different levels of a thyroid lobe in the experiment with thiouracil-fed animals.

Three hundred random tissue point-intersections were made in an average total of 25 high-power fields per section. Point-contacts with the lumens of vessels were considered as part of the stroma, but where the random ocular points coincided with an empty tissue space (other than intrafollicular or intravascular in position), these were omitted as being tissue artifacts. The Masson tri-chrome stain greatly facilitated the studies by defining connective tissue elements from parenchymal cells.

By assuming an approximate equivalent specific weight of parenchymal cell, follicle space fluid, and stromal elements, the percentages of relative tissue components by area were translated into similar porportions on the basis of total gland wet weight. For example, if a gland of 100 mgm. had 35% parenchymal cells, 45% follicle spaces, and 20% stroma by histological cross-sectioned area calculation, it was assumed that the relative weight fractions were 35 mgm., 45 mgm., and 20 mgm. respectively. Some license with accuracy was taken in equating the area percentages of tissue constituents with volume and weight, but the differences in the specific weights of the various tissue components were regarded as insignificant. Since the values are relative and subject to similar artifacts (i.e., shrinkage), these errors were considered to be relatively small.

The total thyroid cell mass per whole gland was then defined as the product of the percentage of thyroid cells per gland and the total wet weight of the gland. Therefore, the RNA concentration per unit weight of cell mass (RNA μg./mgm. thyroid cell mass) can be computed as follows:

$$\frac{\text{RNA } (\mu\text{g./mgm. gland}) \times \text{total gland weight (mgm.)}}{\text{Fraction of gland as cells} \times \text{total gland weight (mgm.)}}$$

$$= \frac{\text{Total RNA } (\mu\text{g.})/\text{thyroid gland}}{\text{Total thyroid cell mass (mgm.)}} = \text{RNA } (\mu\text{g.})/(\text{mgm.}) \text{ thyroid cell mass.}$$

In order to obtain an index of the actual number of cells in thyroid tissue, estimates of relative thyroid cell population density were made by counting the number of thyroid cell nuclei in a standard area of gland median section, using an ocular grating ruled into squares (10). Three separate counts of the nuclei lying within a standard area ($84{,}500\mu^2$) were made on each section (total area, $253{,}000\mu^2$). The average of these values was recorded as the "cell population density factor," or the number of thyroid cell nuclei per $84{,}500\mu^2$ area. This index constitutes an estimate of cell population density of an entire gland and represents a fair sampling of a central thyroid lobe section. As was observed in comparing sections cut at three different levels of one series, there was a remarkable degree of homogeneity of the tissue. The average nuclear size remained quite constant in the various experiments and was therefore not considered a significant variable in these cell population calculations.

Average thyroid cell height measurements were obtained by the ocular micrometer direct technique using oil immersion objectives. A total of twenty-five representative cells, each from one randomly selected intact follicle, was measured in each gland section (11).

Since the structure of the thyroid makes technically difficult an exact isolation for counting of nuclei from tissue homogenates, the average DNA content in the guinea pig diploid nucleus (5.9×10^{-12} g.) was used in calculating the number of cells per gland (12). This value represents the average DNA content of one nucleus from resting tissue with a low mitotic index, so that calculations on more actively dividing thyroid cells (i.e., thyrotropin-treated animals) have only relative significance. The total number of cells in the thyroid was determined by dividing the DNA per whole gland by the assumed average nuclear DNA content (5.9×10^{-12} g.). The average cell content of RNA was then calculated by dividing the RNA per whole gland by the total number of cells of the gland.

The differences in RNA content per cell do not invariably refer to actual changes in concentration of RNA per unit cell mass because of the marked fluctuations in relative cell sizes as indicated by the average cell height determinations. Furthermore since the change in total DNA represents a change in number of cells, the change of the ratio

$$\left(\frac{\text{total cell mass}}{\text{total DNA}}\right)$$

versus controls provides a relative index of cell size.

Chemical Methods

All tissues were kept on ice, weighed immediately, and homogenized in chilled Potter-Elvejhem glass homogenizers with chilled 0.25 M. sucrose. If not immediately used for determination of nucleic acids, the homogenate was kept at −20° C.

Aliquots of tissue were dried to constant weight in all glass apparatus with vacuum, P_2O_5, and heating by boiling toluene.

DNA and RNA were isolated by the Schmidt-Thannhauser-Schneider (13) procedure and quantitated in duplicate by the orcinol reaction for ribose of RNA and diphenylamine reaction for desoxyribose of DNA. Yeast RNA and calf thymus DNA were used as standards. The duplicate analyses of DNA agreed within 3%, and of RNA within 4%. The results agreed with those obtained by spectrophotometric estimation of purine and pyrimidine constituents of RNA and DNA. The Schmidt-Thannhauser-Schneider procedure was compared with Schneider's method (14). The RNA data agreed, but the DNA analyses by the Schmidt-Thannhauser-Schneider technique were about 10% lower than found by Schneider's method. Along with analysis of RNA and DNA in the thyroid tissue, both nucleic acids were determined in the rat liver for control purposes. The data on nucleic acid analysis were expressed as µg./mg. wet tissue. The water content of aliquots of tissue was determined so that the results could be related per unit weight of dry tissue. The data on nucleic acid analysis were also expressed per unit of cell mass.

Because the analyses were made from whole tissue homogenates, the contribution to DNA and RNA by the stroma cells was not determinable. However, this was considered relatively constant since the amount of thyroid stroma changed very little in these experiments. In order to determine the nucleic acid content of colloid, sheep thyroid glands were frozen with CO_2. The tissue was cut in slices 10–15µ in thickness, shaken in chilled saline, and centrifuged in the refrigerated centrifuge. Tissue which was resuspended showed retention of follicular structure but was devoid of colloid. The supernatant-containing colloid was used for DNA and RNA determinations. Since no DNA and an insignificant amount (0.13 µg./equivalent 1 mg. wet tissue) of RNA was found in the colloid, the analysis of nucleic acids of the homogenate can be related to cell mass.

RESULTS

The effects of thyrotropin, thyroxine, and thiouracil on the nucleic acids of the guinea pig thyroid gland

Thyrotropin-treated animals. In the thyrotropin-treated animals, the average wet weight of the thyroid increased 128%, while total cell mass of the gland increased 330%. The cell population density was decreased 19%. The average cell height was increased 246%. The DNA per unit weight of wet tissue was slightly increased (10%) while DNA calculated per unit weight of cell mass was markedly decreased (41%). Finally, the total DNA per gland was increased 150%.

The increase in total cell mass along with a decrease in cell population density indicate hypertrophy of thyroid cells, for as cytoplasm volume increased, the cell and nuclear population densities naturally tended to decrease. Furthermore, the hypertrophy of the cell is evident from the proportionally greater increase in total cell mass (330%) than in total DNA per whole gland

$$\left(\frac{\text{increase in total cell mass} = 330\%}{\text{increase in total DNA} = 150\%} = 2.2\right).$$

Therefore this ratio (2.2) is an index of relative cell enlargement. The hypertrophy of the cells is responsible for a great decrease in DNA per unit weight of cell mass.

The inconsistency of an increase in DNA per unit weight of wet tissue coexisting with a decrease in cell population density, apart from a technical error, could be explained by an increased number of cells with polyploid nuclei, i.e., nuclei undergoing mitosis. This phenomenon does not represent a specific effect of thyrotropin on the nuclear content of DNA of each individual thyroid cell, since it has been observed in other growing tissue, such as in the nuclei of the regenerating liver (15). Therefore, these data suggest that the nuclear content of DNA does not change under thyrotropin stimulation.

The RNA per unit weight of wet tissue was considerably elevated (146%), but the increase in RNA per unit of cell mass was less marked (32%). The increase in total RNA per gland was 463%.

TABLE 1. DATA ON CELL HEIGHT, STRUCTURE, CELL POPULATION DENSITY AND I^{131} UPTAKE OF THE THYROID GLAND

	Average cell height, μ	Thyroid cell %	Follicle space %	Stroma %	Cell population density factor = (number of nuclei/84,500 μ^2)	I^{131} uptake % of dose
Control	3.7 (0.2)	29.1 (1.8)	50.7	20.1	347 (12)	6.7
Thyrotropin 0.5 U.S.P. sc/day for 14 days	12.8 (0.8)	54.7 (3.5)	24.8	20.5	282 (12)	64.8
	+246%	+87%	−51%	+2%	−19%	+867%
l-sodium thyroxine 100 μg. sc/day for 14 days	3.4 (0.3)	33.8 (2.4)	48.4	17.8	374 (12)	1.0
	−8%	+16%	−5%	−11%	+8%	−85%
Control	5.2 (0.7)	30.4 (1.0)	52.9	16.7	257 (13)	4.8
Thiouracil 0.2% in food for 90 days	12.5 (0.4)	45.5 (0.5)	32.2	22.3	241 (10)	18.5
	+140%	+50%	−39%	+34%	−6%	+285%

Figures in parentheses are standard errors.

The increase in total RNA (463%) over the increase in average weight of the gland (128%) in the thyrotropin-treated animals

$$\left(\frac{\text{increase in total RNA} = 463\%}{\text{increase in gland weight} = 128\%} = 3.6\right)$$

appeared much greater than when correlated with the increase (330%) in total cell mass

$$\left(\frac{\text{increase in total RNA} = 463\%}{\text{increase in total cell mass} = 330\%} = 1.4\right).$$

The latter ratio (1.4) serves as an index of the change of RNA concentration per unit weight of cell mass. The average amount of RNA per cell showed an increase of 125%. The increase in RNA per cell is proportionally greater than the increase of protoplasm in the cell

$$\left(\frac{\text{increase in total RNA} = 463\%}{\text{increase in total cell mass} = 330\%}\right)$$

and therefore the concentration of RNA in the thyroid cell is increased compared to the control gland. These data suggest that RNA is increased in the thyroid cell stimulated by exogenous thyrotropin. The increase in RNA is less marked when related to the unit weight of cell mass than to the unit weight of whole tissue. The increase in total RNA is proportionally greater than the increase in total cell mass.

Thyroxine-injected animals. In the thyroxine-injected animals, the average weight of the thyroid was decreased 32% and the total cell mass of the gland decreased 21%. The average cell height was slightly decreased (8%). The cell population density showed a small increase (8%). The DNA per unit weight of wet tissue was increased (14%) while DNA per unit weight of cell mass was not significantly changed (−2%). The total DNA per gland was decreased 23%. The decrease in total cell mass along with slight increase in cell population density suggest a very small decrease in size of thyroid cells. Furthermore the small decrease in size of

TABLE 2. DATA ON WEIGHT, WATER CONTENT, DNA AND RNA OF THE THYROID GLAND

	Thyroid average weight, mg.	Water %	DNA, µg./mg. wet tissue	DNA, µg./mg. cell mass	RNA, µg./mg. wet tissue	RNA, µg./mg. cell mass	RNA/DNA, µg./wet tissue
Control	47.5	74.0	4.73	16.20	1.96	6.71	0.41
Thyrotropin 0.5 U.S.P. sc/day for 14 days	108.5 +128%	78.9 +7%	5.18 +10%	9.47 −41%	4.83 +146%	8.83 +32%	0.93 +127%
l-sodium thyroxine 100 µg. sc./day for 14 days	32.2 −32%	71.3 −4%	5.40 +14%	15.93 −2%	1.16 −41%	3.42 −49%	0.22 −46%
Control	75.9	74.8	4.99	16.42	2.09	6.88	0.42
Thiouracil 0.2% in food for 90 days	314.1 +314%	78.0 +4%	4.80 −4%	10.55 −36%	3.22 +54%	7.08 +3%	0.67 +60%

thyroid cells is evident from the essentially equal decreases in total cell mass (−21%) and in total DNA per gland

$$\left(\frac{\text{decrease in total cell mass} - 21\%}{\text{decrease in total DNA} - 23\%} = 0.9\right)$$

this ratio (0.9) being an index of relative cell size. The decrease in average weight of the thyroid was more due to decreased water and colloid content of the tissue than to atrophy of the cells. The increase in DNA per unit weight of wet tissue is a relative one and is due to the relative increase in cell population density. This is supported by the fact that there was no significant change in DNA per unit weight of cell mass. These data suggest that the nuclear content of DNA does not change in the absence of thyrotropin stimulation.

The RNA per unit weight of wet tissue was markedly decreased (41%) and the decrease in RNA per unit weight of cell mass was similarly 49%. The similarity of these two forms of expression merely shows that the proportions of histological constituents did not change compared to the control gland despite a definite decrease in average weight of the gland (32%). The total RNA per gland decreased 60%. The decrease in total RNA per gland (60%) was greater when compared to the decrease (32%) of average weight of the gland

$$\left(\frac{\text{decrease in total RNA} = 60\%}{\text{decrease in gland weight} = 32\%} = 1.9\right)$$

and this ratio was smaller than when the change of RNA was compared with the change (−21%) in total cell mass

$$\left(\frac{\text{decrease in total RNA} = 60\%}{\text{decrease in total cell mass} = 21\%} = 2.9\right).$$

The latter ratio (2.9) is an index of the change of RNA concentration per unit weight of cell mass. The RNA per cell showed a decrease of 48%. The decrease in RNA per cell is proportionally greater than the decrease of protoplasm in the cell

$$\left(\frac{\text{decrease in total RNA} = 60\%}{\text{decrease in total cell mass} = 21\%}\right)$$

and therefore the concentration of RNA in the cell is decreased when compared to the control gland.

These data suggest, that in the absence of thyrotropin stimulation the RNA is decreased in the thyroid cell. The decrease in RNA was very similar whether related to the unit weight of cell mass or to unit weight of wet tissue. The decrease in total RNA per gland was proportionally greater than the decrease in total cell mass.

Thiouracil-fed animals. In the thiouracil-fed animals, the average weight of the thyroid increased 314%, and the total cell mass of the gland increased 519%. The average cell height increased 140%, the cell population density was not significantly changed (−6%). The DNA per unit weight of wet tissue was not significantly changed (−4%), while the DNA per unit weight of cell mass considerably decreased (36%). The total DNA per gland increased 299%. The increase in cell mass with no significant change in cell population density indicate hypertrophy of thyroid cells. The hypertrophy of thyroid cells is evident from a proportionally greater increase in total cell mass than in total DNA per gland

$$\left(\frac{\text{increase in total cell mass} = 519\%}{\text{increase in total DNA} = 299\%} = 1.7\right).$$

The hypertrophy of the cells is responsible for the decrease in DNA per unit weight of cell mass. The DNA per unit weight of wet tissue and the cell population density were in agreement and their decrease was not significant. Therefore, these results suggest that the nuclear content of DNA is not changed when the secretion of endogenous thyrotropin is increased.

The RNA per unit weight of wet tissue was elevated 54%, but the increase in RNA per unit weight of cell mass of the gland was not significant (3%). The total RNA per gland increased 538%.

The increase in total RNA (538%) compared to the (314%) increase in average weight of the gland

$$\left(\frac{\text{increase in total RNA} = 538\%}{\text{increase in gland weight} = 314\%} = 1.7\right)$$

was larger than when correlated with the increase (519%) in total cell mass

$$\left(\frac{\text{increase in total RNA} = 538\%}{\text{increase in total cell mass} = 519\%} = 1.0\right).$$

The latter ratio of approximate unity indicate that the total RNA increase paralleled the increase in the total mass of protoplasm. The average RNA per cell was increased 60%. However, the increase in RNA per cell was proportionally similar to the increase of protoplasm in the cell

$$\left(\frac{\text{increase in total RNA} = 538\%}{\text{increase in total cell mass} = 519\%}\right)$$

and therefore the concentration of RNA in the thyroid cell has not increased.

These data suggest that RNA is increased in the thyroid cell stimulated by increased secretion of thyrotropin. The increase in RNA is not significant when calculated per unit weight of cell mass; for the increase in total RNA per gland is proportionally equal to the increase in total cell mass.

TABLE 3. DATA ON CALCULATED TOTAL THYROID CELL MASS; DNA AND RNA IN WHOLE WET TISSUE, CELL MASS AND IN INDIVIDUAL CELL

	Calculated total thyroid cell mass, (mg.) = (% of cells × aver. gland weight)	DNA/mg. gland $\times 10^{-6}$g.	Total DNA/ gland $\times 10^{-6}$g.	RNA/mg. gland $\times 10^{-6}$g.	RNA/mg thyroid cell mass $\times 10^{-6}$g.	Total RNA /gland $\times 10^{-6}$g.	Calculated total No. of thyroid cells $\times 10^{6}$	RNA/cell $\times 10^{-12}$g.
Control	13.8	4.73	225	1.96	6.7	93.1	38	2.4
TSH 0.5 U.S.P. sc/day for 14 days.	59.4 +330%	5.18 +10%	562 +150%	4.83 +146%	8.8 +32%	524 +463%	95 +150%	5.5 +125%
l-sodium thyroxine 100 mg. sc/day for 14 days	10.9 −21%	5.40 +14%	174 −23%	1.16 −41%	3.4 −49%	37.4 −60%	30 −23%	1.3 −48%
Controls	23.1	4.99	379	2.09	6.9	159	64	2.5
Thiouracil 0.2% in food for 90 days.	142.9 +519%	4.80 −4%	1508 +299%	3.22 +54%	7.1 +3%	1011 +538%	256 +299%	4.0 +60%

The RNA/DNA ratio was increased 127% in thyrotropin-treated and 60% in thiouracil-fed animals, while in thyroxine-injected animals it was decreased 46%. The same changes in RNA/DNA ratio are obtained if RNA and DNA are expressed per unit of cell mass.

The uptake of I^{131} by the thyroids was greatly increased in thyrotropin-treated and very decreased in the thyroxine-injected animals. The increased uptake in thiouracil-fed animals was due to increased trapping of inorganic I^{131}, without formation of significant amounts of protein-bound I^{131} (0.2%), and by chromatography of trypsin-digested thyroid homogenate practically all I^{131} was found to be iodide.

DISCUSSION

The data indicate that histological structure should be considered in the evaluation of chemical assays on a non-solid organ, such as the thyroid gland. When expressed in the conventional manner per unit wet weight of thyroid tissue or per whole gland, chemical analyses may be misleading. For example, the cell mass in the experimental thyroids of this study ranged from a low of 29.1% (control) to a high of 54.7% (thyrotropin-treated) so that a direct comparison of chemical assays on one mgm. samples of these two glands would be made upon unequal samples of protoplasm. This is also evident from the differences of percentage changes of RNA as expressed per unit weight of whole tissue, per unit weight of cell mass and per whole gland and illustrates how comparisons are actually made on different volumes of protoplasm.

These results suggest that thyrotropin does not affect the nuclear content of DNA. The change in DNA per unit weight of wet tissue or per whole gland under the stimulation of increased amount of exogenous or endogenous thyrotropin reflects an absolute increase in the number of cells. The increase in concentration of DNA in the absence of thyrotropin (in thyroxine-injected animals) corresponds with the relative increase in number of cells due to shrinkage of follicles and stroma, while the decreased DNA content per whole gland suggests a decrease in total number of cells. The greater increase in DNA per whole gland of thiouracil-fed animals than in thyrotropin-treated animals (229%:150%) most probably means that hyperplasia of thyroid cells is greater in the former than in the latter group. The polyploidy of the nuclei is not significantly responsible for the increase of total DNA in thiouracil-fed animals because Santler (16) observed six mitoses/300,000 cells in control, and only one mitosis/800,000 cells in thyroid glands of rats fed thiouracil for 30 days. Since in this experiment animals were fed thiouracil for 90 days, probably a relatively small number of cells was undergoing mitosis at the time of sacrifice. On the other hand, the greater hypertrophy of cells in the thyrotropin-treated group than in the thiouracil-fed animals is corroborated by a correlation of change in total cell mass of the gland with the change in total DNA per gland,

$$\left(\frac{\text{increase in total cell mass} = 330\%}{\text{increase in total DNA} = 150\%} = 2.2\right)$$

in the thyrotropin-treated, and

$$\left(\frac{\text{increase in total cell mass} = 519\%}{\text{increase in total DNA} = 299\%} = 1.7\right)$$

in the thiouracil-fed animals. Therefore, these data suggest that during the short but very intensive stimulation of the thyroid by thyrotropin, the hypertrophy of cells contributed relatively more than hyperplasia to the enlargement of the gland.

These data also suggest that the stimulation of the thyroid with increased amounts of exogenous and endogenous thyrotropin is intimately associated with an increase of total RNA per gland. This is also evident by a great decrease in RNA in the absence of thyrotropin stimulation as observed in thyroxine-injected animals.

The effect of thyrotropin on RNA is much less pronounced if the RNA is related to the unit weight of cell mass of the gland rather than to the unit weight of whole, wet tissue. The data also show that the increase in RNA was smaller with prolonged stimulation by endogenous thyrotropin than in short-term and intensive stimulation with exogenous thyrotropin. The smaller increase in the RNA of thiouracil-fed than of thyrotropin-treated animals could conceivably be due to an inhibitory effect of thiouracil *per se* on the synthesis of RNA, as has been observed in tobacco mosaic virus (17) and bacterium coli (18). However, since RNA is related to protein synthesis and growth of the cells, it is difficult to reconcile this explanation with the fact that the increase in average weight of the thyroid was greater in thiouracil than in thyrotropin-treated animals. Therefore, it is probable that thiouracil *per se* does not significantly inhibit the synthesis of RNA in the thyroid gland.

The present observations of: 1. a relatively constant DNA content of individual nuclei; 2. increases or decreases of RNA in activated or inactivated thyroid cells respectively were not reported in previous studies on nucleic acids in the thyroid gland by Dempsey and Singer (1); Reich (2); Roels (4); and Rerabek and Rerabek (5); and Van Erkelns (7). The results of present studies are essentially in agreement with the observations of Suzuki (6); Alfert, Bern and Kahn (3); and Fiala, Sproul and Fiala (8).

Close quantitative comparisons of the results of previous and present studies are not possible because different laboratory techniques were used and in none of the previous studies were correlations made between the chemical change of nucleic acids and the change of the structure of the thyroid gland.

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LIMITATIONS OF THE CHICK RADIOPHOSPHORUS UPTAKE ASSAY AND THE CHICK RADIOIODINE DEPLETION ASSAY FOR THYROTROPIC HORMONE WHEN APPLIED TO BLOOD AND URINE[1,2]

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ABSTRACT

The chick thyroid P^{32} uptake and I^{131} depletion methods for the bioassay of anterior pituitary thyrotropic hormone (TSH) have been applied to the measurement of TSH in human serum and urine. Although there are detectable levels of TSH in sera from some patients with myxedema, hyperthyroidism, or exophthalmos, the levels in normal individuals are too low to measure accurately. The ultrafiltrate residue of urine contains a non-hypophyseal factor which stimulates the uptake of P^{32} by the chick thyroid, which imposes limitations on the application of the P^{32} uptake assay to urine. Urine ultrafiltrate residue also contains a non-hypophyseal factor which interferes with the effect of TSH on I^{131} depletion in the chick thyroid. Assay of TSH in human serum or urine by these methods will require, therefore, either concentration of TSH from serum or elimination of interfering factors from urine.

THE uptake of radiophosphorus (P^{32}) by the thyroid gland of the chicl and the depletion of radioiodine (I^{131}) from the chick thyroid hav been developed into simple and sensitive procedures for the bioassay c anterior pituitary thyrotropic hormone (TSH) (1, 2, 3, 4). These methoc are adaptable to a 4- or 6-point bioassay design (5) which may then b applied to the measurement of TSH in human serum and urine. Substance interfering with the bioassay of TSH have been found in serum by Poste (6) and in urine by Greenspan *et al.* (7) and Starr *et al.* (8). This repor deals with the limitations of the chick P^{32} uptake assay and the chick I^{1} depletion assay, as applied to blood and urine, because of the presence o nonspecific reactive substances and interfering substances.

MATERIALS AND METHODS[3]

Blood: Fresh or frozen sera, or blood specimens prepared by the method of de Roberti (9), were examined.

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[1] This work was supported in part by Grant Number A-568 from the National Institut of Arthritis and Metabolic Diseases, Public Health Service.

[2] Presented to the American Goiter Association June 18, 1958.

[3] TSH standard preparation Lot No. 6, used in these experiments, was kindly sup plied by Armour Laboratories, Kankakee, Illinois.

Urine: Twenty-four hour specimens of urine, collected under refrigeration, were ultrafiltered through a 2% collodion membrane according to the method of Gorbman (10). The membrane was removed with anhydrous alcohol-ether and the dried ultrafiltrate residue (UFR) was dissolved in saline prior to assay. In the presence of a small amount of carrier protein, quantitative recovery of purified TSH was achieved by ultrafiltration. The dose of urine UFR is expressed in "hours," *i.e.*, one hour of urine UFR represents 1/24 of a daily urine collection. Twenty-four hours of human urine UFR contained about 50 mg. of protein, as well as small amounts of Na, K, Mg, and Ca. Doses of 4 hours of human urine UFR produced no evident toxic effects in recipient chicks.

The P^{32} uptake assay: The details of this assay, based upon the uptake of P^{32} by the chick thyroid 6 hours after intracardiac administration of TSH or test substance, have been reported (1). The response is expressed as $\sqrt{t}$, where t equals the time required to count 1024 counts per thyroid. Increased P^{32} uptake induced by TSH is indicated by decreased $\sqrt{t}$. The useful range of the assay is 0.25 to 5 milliunits of TSH (1 milliunit, abbreviated "mu." equals 0.001 U.S.P. unit of TSH). The precision of a series of assays, using groups of 5 chicks per dose, was indicated by a mean lambda of 0.34.

The I^{131} depletion assay: The method of Bates and Cornfield (4), based upon the depletion of I^{131} from the thyroid glands of chicks treated with thryoxine and propylthiouracil, was found satisfactory only with chicks having a high spontaneous uptake of I^{131}.[4] The response is expressed as per cent of the initial content of I^{131} remaining 24 hours after subcutaneous administration of test substances. Increased TSH potency is indicated by decreased per cent I^{131} remaining. The useful range of the assay was 1 to 16 mu. TSH, and with groups of 5 chicks per dose the mean lambda for a series of assays was 0.22.

Other methods of study: Twenty-four hour I^{131} uptakes were determined in thyroxine treated chicks after 2 days of administration of test substances. Histological examination of chick thyroids was made after 3 days of administration of test substances. These techniques were comparatively insensitive to TSH but were useful to demonstrate substances interfering with TSH.

RESULTS

Attempts to assay TSH in serum

Limitations of the P^{32} uptake assay: We were unable to detect TSH activity in serum from normal subjects, patients with Graves' disease, or patients with malignant exophthalmos. TSH was not detectable in blood processed by the method of de Robertis (9) nor in serum protein fractions prepared by continuous flow paper electrophoresis according to the method of Durrum (11) (Table 1). Although the P^{32} uptake assay will detect 0.25 mu. of TSH, intracardiac injection of test substances limits the injection volume to 0.1 cc., so that the minimal detectable amount of TSH in unconcentrated serum would be 2.5 mu. per ml. Application of this assay technique to untreated serum is limited to specimens containing very high levels of TSH.

Limitations of the I^{131} depletion assay: This assay will usually detect 1 mu. of TSH; and since a maximum of 2 ml. of serum can be administered

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[4] White leghorn cockerels, obtained from Kimber Hatchery, Niles, California, were satisfactory until June 1958, when I^{131} uptake diminished due to addition of iodide to the diet.

TABLE 1. THE EFFECT OF HUMAN SERUM AND BLOOD FRACTIONS ON P^{32} UPTAKE OF CHICK THYROID

Source of serum or blood	Dose,† ml./chick	P^{32} uptake response*			
		Serum or blood $\sqrt{t}+2$ SE	Control $\sqrt{t}+2$ SE	TSH standard	
				Dose, mu.‡	Response, $\sqrt{t}+2$ SE
Normal serum	0.1	11.94 ±0.82	12.27 ±1.21	0.5	10.15 ±1.13
Exophthalmos serum	0.1	10.80 ±0.32	10.61 ±1.19	0.75	7.89 ±0.67
Hyperthyroid serum	0.1	12.59 ±0.63	12.30 ±1.05	0.5	10.93 ±0.47
Normal blood,§ 85% acetone precipitate	0.5	10.15 ±1.00	11.57 ±1.30	2.0	7.29 ±0.82
Normal serum:‖					
Albumin	0.13	10.01 ±0.20	11.07 ±1.28	0.5	11.00 ±1.21
$\alpha+\beta$ Globulin	0.13	10.92 ±0.60		1.5	8.67 ±1.02
γ Globulin	0.17	11.21 ±1.00		4.5	6.90 ±1.11

* Response expressed as $\sqrt{t}$, where t equals time to count 1024 cts. per thyroid. *Values represent mean ±2 standard errors for groups of 5 chicks.*

† Dose of fresh serum, or the amount of blood or serum represented by 0.1 ml. of concentrate.

‡ *1 mu. TSH equals 0.001 U.S.P. unit of TSH.*

§ Blood processed by method of de Robertis (9).

‖ 2.5 ml. serum fractionated by continuous flow paper electrophoresis method of Durrum (11).

to the chick subcutaneously, the minimal detectable concentration of TSH in serum would be 0.5 mu. per ml. The concentration of TSH in serum would have to be considerably higher than this to permit application of a 4-point bioassay procedure. Table 2 shows the results of a series of assays in which the I^{131} depletion response of 2-ml. portions of serum from vari-

TABLE 2. ACTIVITY OF SERA FROM VARIOUS PATIENTS IN THE I^{131} DEPLETION ASSAY

Diagnosis	Patient		I^{131} depletion response*			
	Sex	Age	Control, % R ±2 SE*	TSH, 1 mu. % R ±2 SE†	Serum, 2 ml. % R ±2 SE	P‡ value
Normal	F	36	86.4 ±3.0	66.5 ± 5.8	82.8 ± 2.8	>.05
	F	31	79.5 ±3.2	78.5 ± 3.4	80.9 ± 5.0	>.05
	F	44	88.8 ±4.0		78.6 ± 7.0	>.02, <.05
	M	38	84.6 ±2.4	72.3 ± 8.2	72.9 ±11.0	.05
	M	30	92.2 ±7.0	77.6 ± 8.2	83.3 ± 1.5	.05
Myxedema	F	50	84.6 ±2.4	72.3 ± 8.2	76.7 ± 7.4	.05
	F	55	100.8 ±6.4	92.4 ± 6.4	80.4 ± 5.2	.01
	F	50	85.6 ±3.2	76.5 ±11.4	75.0 ±11.4	>.05
	F	52	86.5 ±4.2	75.4 ± 5.8	74.2 ±11.2§	>.05
Graves' Disease	F	38	100.8 ±6.4	92.4 ± 6.4	90.4 ± 6.8	>.05
	F	55	100.8 ±6.4	92.4 ± 6.4	84.8 ± 5.6	<.01
Malignant Exophthalmos	M	50	92.0 ±3.4	84.1 ± 2.6	76.7 ± 1.5	<.01
	M	30	74.7 ±7.8	77.6 ±12.4	67.7 ±11.5	>.05
Pituitary Myxedema	F	71	85.6 ±3.2	76.5 ±11.2	84.6 ± 3.8	>.05

* Response (%R) expressed as % initial thyroid content of I^{131} remaining 24 hours after test substance, mean ±2 standard errors for groups of 5 chicks.

† This is a single point from the TSH dose response curve presented to indicate the sensitivity of the assay. 1 mu. TSH equals 0.001 U.S.P. unit of TSH.

‡ P value for comparison of serum group with control group.

§ Response to 0.6 ml. serum.

ous patients was compared with a TSH standard curve run simultaneously. Sera from normal subjects revealed barely significant I^{131} depletion activity. Although it is not obvious from the individual assays, it can be shown by pooling the data that sera from patients with myxedema, Graves' disease, and malignant exophthalmos were more active, but the activity was still not high enough to permit utilization of a 4-point bioassay procedure. Thus, even with the increased precision achieved by use of larger groups of animals, it is apparent that the I^{131} depletion assay is also limited to sera containing high levels of TSH.

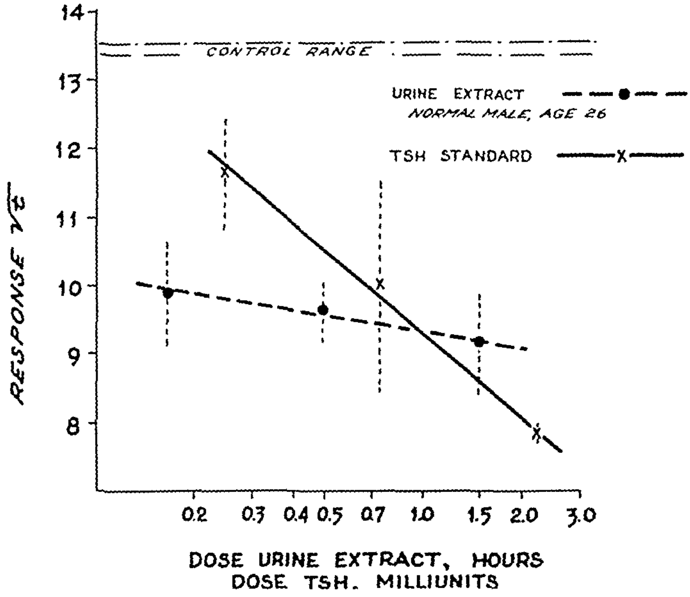

FIG. 1. Effect of TSH and urine UFR on P^{32} uptake assay. Each point represents mean response ±1 standard deviation for group of 5 chicks. In this and in succeeding charts, the dose of urine UFR is expressed in hours, where 1 hour equals 1/24 of a daily urine collection.

Attempts to assay TSH in urine ultrafiltrate residue

Limitations of the P^{32} uptake assay: We have found that urine UFR from all subjects tested stimulated the uptake of P^{32} by the chick thyroid. Specimens of urine UFR from normal individuals were quite active in the P^{32} uptake assay, but the dose-response curves were flat (Fig. 1). In 60 assays of urine UFR from various patients, the slopes of the urine curves were significantly less than the slopes of the standard TSH curves. This suggested that the factor in urine UFR active in the P^{32} uptake assay was different from TSH or that some interfering factor was present. Specimens of urine UFR from 5 patients with clinical and laboratory evidence of panhypopituitarism were found to have considerable activity in this assay (Table 3). In these subjects, we did not have autopsy proof of the completeness of hypophysectomy. However, we have assayed samples of

TABLE 3. EFFECT OF URINE UFR FROM PATIENTS WITH PANHYPOPITUITARISM ON THE P^{32} UPTAKE OF CHICK THYROIDS

Patient	Clinical diagnosis*	Control response, $\sqrt{t} \pm 2$ SE†	TSH dose,‡ mu.	TSH response, $\sqrt{t} \pm 2$ SE†	UFR dose, "hrs."	UFR response, $\sqrt{t} \pm 2$ SE†
Don	Craniopharyngioma	15.23 ± 0.81	0.75	11.49 ± 1.24	3.0	11.08 ± 1.34
Tak	Craniopharyngioma	10.41 ± 0.66	0.75	9.13 ± 0.71	1.5	8.33 ± 0.68
McC	Chromophobe adenoma	13.61 ± 1.91	0.75	10.73 ± 1.21	1.5	8.95 ± 0.61
Bas	Hypophysectomy§	13.71 ± 0.75	0.75	9.67 ± 1.19	1.5	7.86 ± 1.13
Zwy	Hypophysectomy§	13.71 ± 0.75	0.75	9.67 ± 1.19	1.5	9.67 ± 1.27

* All patients had clinical and laboratory evidence of thyroid insufficiency with panhypopituitarism.

† Response expressed as $\sqrt{t}$, where t equals time required to count 1024 cts. per thyroid, ±2 standard errors. Groups of 5 chicks per dose.

‡ This is a single point from the TSH dose response curve presented to indicate the sensitivity of the assay.

§ Patients with advanced breast carcinoma hypophysectomized by irradiation with deuteron beam. Specimens were obtained through the kindness of Dr. J. N. Lawrence, Donner Laboratory, University of California, Berkeley, California.

urine UFR from normal and totally hypophysectomized dogs[5] and have found considerable activity in each. The dose-response curves from such an assay are presented in Figure 2. Urine UFR from both the normal and the hypophysectomized dog contained essentially the same activity in the P^{32} uptake assay. Within the range tested, there was no increase in response with increased dosage. This effect is considerably different from that of TSH. These findings suggest that there is in urine UFR a nonhypophyseal factor which stimulates the uptake of P^{32} by the chick thyroid. This greatly interferes with the application of the P^{32} uptake assay to the study of TSH in urine.

Limitations of the I^{131} uptake assay: Urine UFR from normal subjects was consistently active in the I^{131} depletion assay, whereas urine UFR from patients with panhypopituitarism was inactive. However, the dose response curve of human urine UFR in this assay was invariably flatter than the TSH curve (Fig. 3). Furthermore, when urine UFR was administered to the chick in a separate site but simultaneously with TSH, the resultant I^{131} depletion dose response curve was significantly modified (Fig. 3). These findings indicated the presence of a factor in normal human urine UFR which interfered with the effect of TSH upon the discharge of I^{131} from the chick thyroid gland. Urine UFR from normal and hypophysectomized dogs was similarly studied. Urine UFR from normal dogs was usually active in the I^{131} depletion assay although the dose-response curve was flat. Urine UFR obtained from hypophysectomized dogs was consistently inactive. This confirmed our observations on human urine UFR, and also implies that the I^{131} depletion response is probably more specific for TSH than the P^{32} uptake response. When urine UFR from normal or hypophysectomized dogs was administered to the chick in separate sites

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[5] These specimens were kindly supplied by Dr. W. F. Ganong, Department of Physiology, University of California School of Medicine.

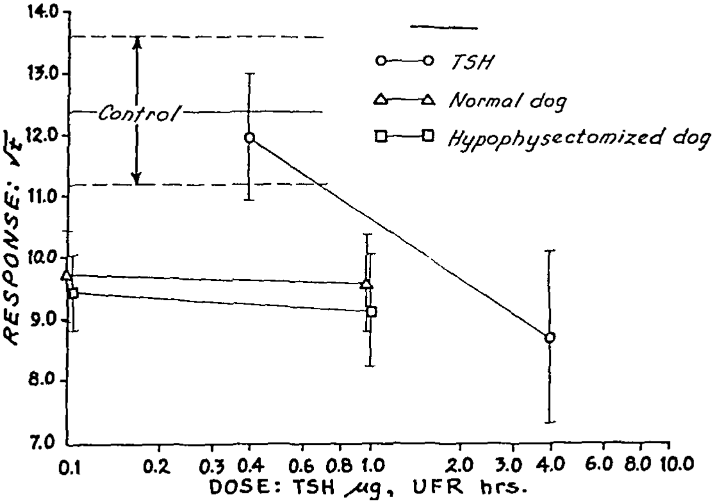

FIG. 2. Effect of urine UFR from hypophysectomized and normal dogs in P^{32} uptake assay. Each point represents mean ±1 standard deviation for group of 5 chicks.

but simultaneously with standard TSH, the expected TSH-induced I^{131} depletion response was partially inhibited (Fig. 4). Although in this experiment there was no spontaneous TSH-like activity in the urine UFR from either the normal or the hypophysectomized animal, 2 hours of urine UFR clearly interfered with the activity of 4 mu. of TSH. A similar type of interference with TSH action was obtained when I^{131} uptake was studied. In chicks with a low spontaneous I^{131} uptake,[c] administration of 50 mu.

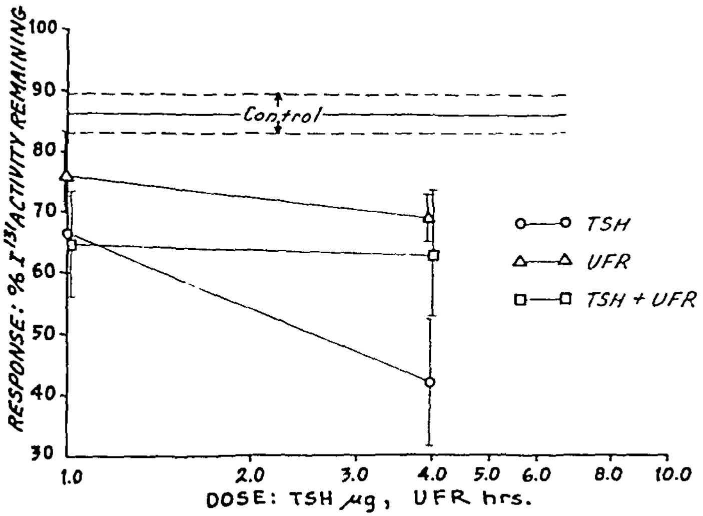

FIG. 3. Separate and combined effects of normal human urine UFR and TSH in I^{131} depletion assay. Each point represents mean ±1 standard deviation for group of 5 chicks.

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[c] White Leghorn cockerels with low I^{131} uptakes were obtained from Poehlmann Hatcheries, Petaluma, California.

of TSH induced a significant increase in uptake. This effect of TSH was completely blocked by the simultaneous administration of 2 hours of urine UFR from normal or hypophysectomized dogs (Fig. 5). Comparable effects were obtained with urine UFR from normal humans.

Urine UFR from human subjects was also able to block the effect of TSH on the histological structure of the chick thyroid. In this experiment, groups of 6 chicks received daily injections of 20 mu. of TSH, or 2 "hours" of human urine UFR, or both, for three days. Twenty-four hours after the last injection the thyroids were removed for histological examination.

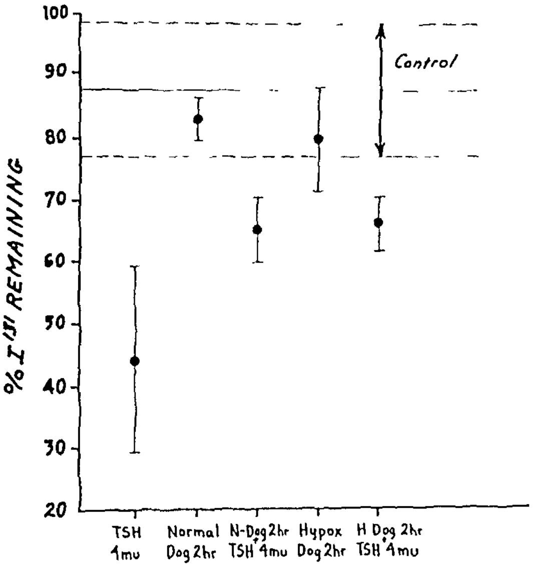

FIG. 4. Effect of urine UFR from normal and hypophysectomized dogs in the I^{131} depletion assay. Each point represents mean ± 1 standard deviation for a group of 5 chicks.

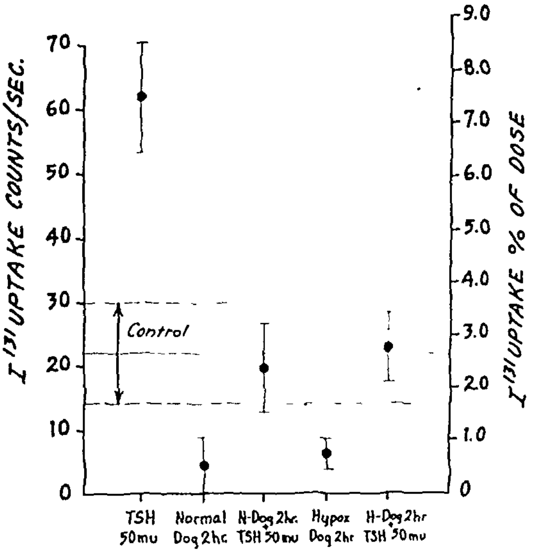

FIG. 5. Effect of urine UFR from normal and hypophysectomized dogs on the I^{131} uptake of chick thyroids. Each point represents mean ± 1 standard deviation for a group of 6 chicks.

Representative sections are presented in Figure 6. TSH induced marked cellular hyperplasia and loss of colloid. This was completely blocked by the simultaneous administration of urine UFR.

These data clearly indicate that there is in human and animal urine a factor which interferes with the action of TSH on the chick thyroid gland. Since it is present in urine from hypophysectomized animals, it is not of hypophyseal origin. The presence of this interfering factor limits the application of the I^{131} depletion assay to urine.

Attempts to separate TSH from interfering substances in human urine UFR

Extensive studies have been made in an effort to separate TSH from interfering substances in urine UFR. No separation of these two activities could be made by treatment of urine UFR with heat, pepsin hydrolysis, sodium periodate, acid hydrolysis, 10% trichloracetic acid extraction,

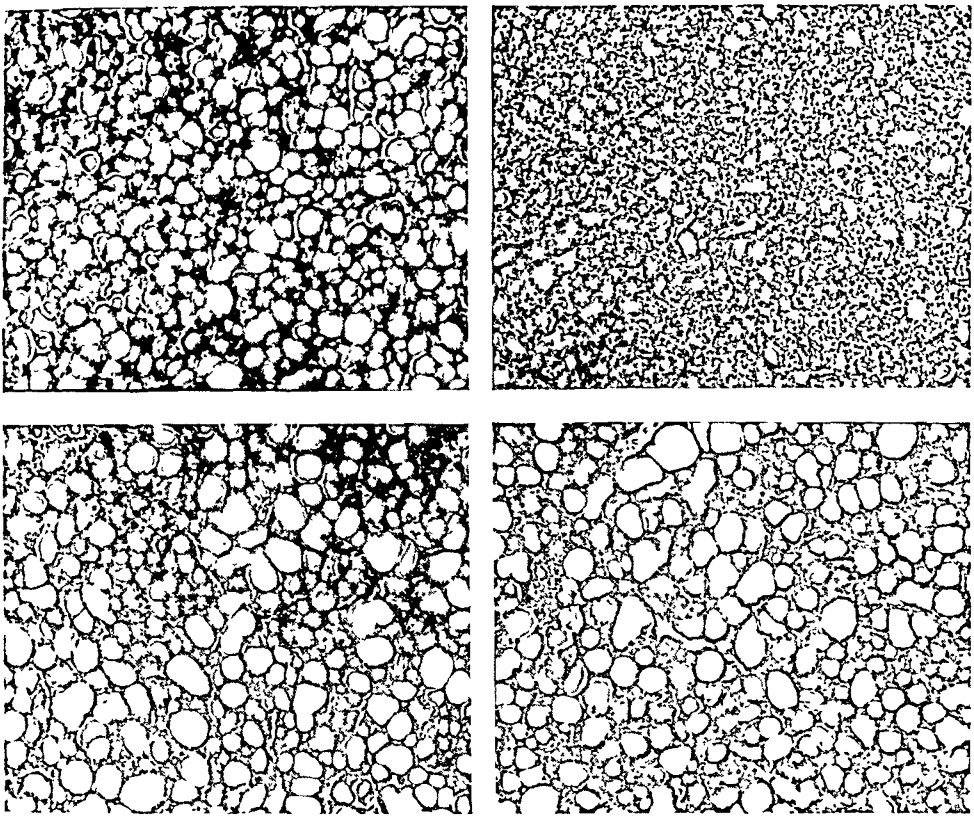

FIG. 6. Effect of TSH and human urine UFR on the histological structure of the chick thyroid. (Hematoxylin and eosin stained sections magnified 220×.) 1. (Upper left) Control 2. (Upper right) 0.02 U.S.P. units of TSH daily for 3 days. 3. (Lower left) 2 hours of UFR daily for 3 days. 4. (Lower right) 0.02 U.S.P. units of TSH plus 2 hours of UFR daily for 3 days.

acid acetone or alkaline acetone fractionation, or separation between n-butanol and p-toluenesulfonic acid (12). We have recently applied the method of Bates and Condliffe (13), using decreasing concentrations of ethanol saturated with NaCl, to elute a mixture of urine UFR and filter aid (Hyflo Super-cel[7]). Preliminary data indicate that bovine TSH may be recovered at a concentration of 65% to 70% ethanol saturated with NaCl. Urine UFR also yields an active fraction eluted at 70% ethanol-NaCl. Further studies are in progress to characterize the relative concentrations TSH and TSH interfering substances in these fractions.

DISCUSSION

Our data suggest that the levels of TSH in normal sera are below 0.5 mu. per ml., confirming the reports of Gilliland Strudwick (3), DiGeorge *et al.* (14), and McKenzie (15). It is possible that high serum TSH levels in patients with myxedema and exophthalmos can be determined by the

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[7] Kindly supplied by Johns-Manville Company, New York 16, New York.

I^{131} depletion assay in a 4-point system, but the levels found in normal individuals are too low to measure by this method. We have evidence that large amounts of inactive serum protein will flatten the dose response curve of purified TSH in both the P^{32} uptake assay and the I^{131} depletion assay. In addition, larger amounts of serum will probably reveal evidence of the factor interfering with TSH described by Postel (6). Isolation of TSH from serum may be difficult since Querido has reported that 2 sera fractionated by the Cohn technique yielded TSH activity in different fractions (16). Further study of serum fractionation is indicated, as is exploration of more sensitive methods of TSH bioassay, such as I^{131} discharge in the mouse as reported by McKenzie (15) or I^{131} uptake of tissue culture preparations of guinea pig thyroid recently described by Bottari (17).

The presence of a non-hypophyseal factor in human and dog urine UFR which is active in the P^{32} uptake assay is quite surprising. The stimulation of the uptake of P^{32} in chick thyroid has been considered a specific effect of TSH (1, 2). Indeed, the only substance other than TSH and urine UFR active in this assay has been cobalt (18). Studies on the distribution of P^{32} in various phosphate-containing fractions of chick thyroid tissue processed by the method of Schneider (19) have revealed differences in the effects of these 3 substances. In these studies, chick thyroid glands were fractionated into acid soluble phosphorus (ASP), lipid soluble phosphorus (LSP), nucleic acid phosphorus (NAP), and phosphoprotein (PP). TSH markedly increased P^{32} uptake into all of these fractions. Cobalt induced a marked rise in P^{32} content of the ASP fraction with slight depletion of P^{32} from the other fractions. Human urine UFR, like TSH, induced significant increments of P^{32} into all fractions, but there was a somewhat greater rise in the P^{32} content of the ASP fraction, and a lesser rise in P^{32} content of NAP. Thus the effects of urine UFR were intermediate between those of cobalt and TSH. The nature of the factor in urine UFR inducing this reaction will require further study. The presence of this factor in urine UFR presents, however, a definite limitation in the use of the P^{32} uptake assay as applied to urine.

The presence of a factor in urine which interferes with the action of TSH is of great importance in evaluating human TSH bioassay data. Since this factor is present in the urine of hypophysectomized dogs, it is not an inactivated or acetylated TSH with blocking effect, as described by Sonenberg and Money (20). It is present in urine extracts prepared by acetone precipitation (9), indicating that it is not an artifact induced by ultrafiltration. Data on urine levels of TSH reported by Bloch-Michel (21) and Currie (22) may have to be corrected for the presence of an interfering substance. This factor interfering with TSH is also of considerable theoretical interest. Although it may represent some non-specific toxic effect of serum or urine, it could represent an extrapituitary mechanism for modifying thyroid function. If it can be separated from TSH in serum or urine, we shall be able to determine more precisely its role in thyroid physiology.

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THE ENDOCRINE SOCIETY

1959 Annual Meeting

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, June 4, 5, 6, 1959.

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5th at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1st 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

4. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.
5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

THE AYERST AND THE SQUIBB FELLOWSHIPS

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

THE SCHERING AND THE UPJOHN SCHOLARS OF THE ENDOCRINE SOCIETY

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1958.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1958.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

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NEUROSECRETORY ACTIVITY IN RATS UNDER CONDITIONS OF CONTINUOUS LIGHT OR DARKNESS

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ABSTRACT

Cytological study of the posterior pituitary and hypothalamic areas of male and female rats kept under usual laboratory conditions (controls), in continuous light, or in constant darkness for eight weeks revealed differences in neurosecretory activity. While the paraventricular nuclei seemed to be unaffected by the varied light treatment, the cells of the supraoptic nuclei were largest and appeared to most active in the lighted rats, smallest and least active in the rats housed in darkness. The amount of neurosecretory material in the hypothalamo-hypophysial tract in two areas, namely, in the region ventral to the supraoptic nuclei and in the median eminence, was estimated from serial sections. It was apparent that Gomori-positive substance was most abundant and occurred in larger clumps along the hypothalamo-hypophysial tract in the continuously lighted rats. It was least in amount in the rats kept in darkness. The neurosecretory material in the posterior lobe was plentiful in the controls, but was reduced in both the other groups. The Herring bodies in the lighted animals were very small.

In light-treated rats large amounts of neurosecretory material appeared to be moving along the neurosecretory axones, while the pars nervosa of such animals was relatively depleted of Gomori-positive substance. This suggests that under the influence of added lighting, the rat both secretes and releases increased amounts of neurosecretory material.

At least a partial separation in function between the paraventricular nuclei and the supraoptic nuclei was indicated by the difference in response of these two nuclei.

PREVIOUS work has shown that light is an important extrinsic factor in the regulation of reproductive activity in many vertebrates. There is also considerable evidence that added lighting increases the secretion and release of the follicle stimulating hormone (FSH) of the anterior pituitary in the rat. Continued exposure to light induces precocious sexual

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maturity, followed in the female by periods of persistent estrus (1, 2, 3). Study of several mammals indicates that the central nervous system plays a role in these phenomena, since an intact retina and optic nerve are essential in the transmission of the light stimulus (4). More recently evidence has been presented which indicates that a neurosecretory link between the anterior pituitary and the hypothalamus is also involved (5). The possibility that the neurosecretory cells of the supraoptic and paraventricular nuclei are the source of such a neurosecretion is supported by anatomical studies showing that axones from the neurosecretory cells constituting these nuclei are closely associated in the median eminence with capillaries which drain into portal vessels supplying the anterior pituitary (6, 7). In addition, work on stalk-sectioned animals (8, 9) and with pituitary grafts (10) shows that a vascular connection between the median eminence and the anterior hypophysis is necessary for the maintenance of cyclic reproductive activity.

In view of the possible importance of the supraoptic nuclei and the paraventricular nuclei in governing some normal secretory functions of the anterior pituitary, a study of their cytology as well as that of the hypothalmo-hypophysial tract in rats housed under conditions of artificially regulated illumination was undertaken.

METHODS

Twenty-two-day old male and female rats were placed in cages and a) exposed to constant illumination from 100w bulbs, b) kept under usual laboratory conditions (12 hours of light, 12 hours of darkness), or c) housed in nearly complete darkness. Eight weeks later the animals were killed instantly, organ and body weights were recorded, and the ovaries were inspected for gross structural features before fixation. The pituitary glands and the hypothalamic area of the brains were fixed in Bouin's fluid. The hypothalamic area was sectioned serially at 10 μ and all sections were mounted. The sections were taken in the frontal plane in fourteen rats and in the sagittal plane in six. All the pituitaries were sectioned at 3μ in the horizontal plane. Three staining methods were employed: 1) Gomori's aldehyde fuchsin (after 1 minute's oxidation with 0.3% potassium permanganate solution and bleaching with 2.5% sodium bisulfite), 2) Gomori's aldehyde fuchsin followed by Halmi's counter-stain (11)[2], and 3) Gomori's chrome-alum-hematoxylin and phloxine (12). Aldehyde fuchsin and chrome-alum-hematoxylin make possible the visualization of neurosecretory material and/or its carrier (6). The dyes used vary in staining capacity with age and temperature, hence only slides stained simultaneously were compared.

Vaginal smears of continuously lighted rats and those housed under regular laboratory conditions were taken daily for two-and-a-half weeks prior to necropsy.

A study of the cytological changes found in the pars distalis in these animals will be reported later.

RESULTS

The hypothalamus in 20 male animals (7 controls, 7 kept in darkness, 6 maintained under constant light) was examined. No difference in the appearance or apparent secretory activity of the cells of the paraventricular nuclei was detected when the three groups of animals were compared. In every instance many of these cells were well filled with neurosecretory granules, and substantial amounts of neurosecretory material were consistently present in the axones leading away from the nuclei. In contrasting fashion the cytology of the supraoptic nuclei varied with the exposure to light. In the control rats the cells of these nuclei were found to be much like those of the paraventricular nuclei except for the more frequent appearance of small, often spindle-shaped cells with small nuclei and containing in the cytoplasm very coarse Gomori-positive granules having the appearance of hyaline material. These cells gave an impression of secretory inactivity. Of particular interest was the difference in the frequency with which these "inactive cells" occurred in each of the three groups. They were few in number in all the lighted rats, intermediate in incidence in the control group and abundant in 6 of the 7 rats housed in darkness. Cells of large size, displaying the characteristics of intense secretory activity, were most evident in the lighted group and sparsely present in the group housed in darkness (Figs. 1, 2). Their abundant cytoplasm contained in the periphery many fine neurosecretory granules, while clustered about the nuclei were coarser granules of similar staining properties. The nuclei were vesicular, with a conspicuous nucleolus.

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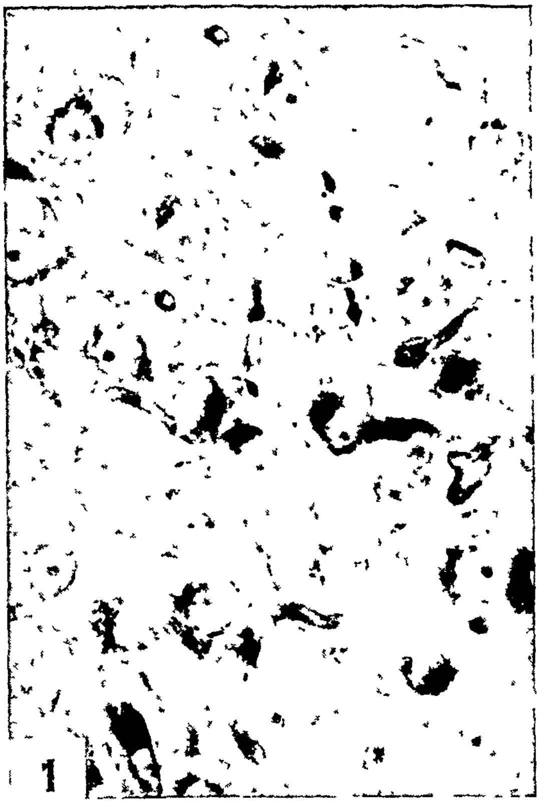
[2] The concentration of light green used was .4% rather than .2%.

The amount of neurosecretory material passing from the hypothalamic nuclei to the pars nervosa was estimated in serial sections. The estimation was made difficult by the fact that the neurosecretory content detectable at different levels varied considerably. Moreover, the neurosecretory products emanating from different hypothalamic nuclei cannot be distinguished in the tractus hypophysius, since axones from the paraventricular nuclei run along beside those of the supraoptic nuclei through much of this area. Mindful of these conditions and limitations, we made comparisons of the neurosecretory material found in the hypothalamo-hypophysial tract at two points; namely, just ventral to the supraoptic nuclei (Figs. 3, 4, 5) and in the median eminence (Figs. 6, 7, 8). Though the distinctions were not always sharp, it was evident, as the illustrations faithfully depict, that at both levels the greatest amount of secretion was found in lighted rats, the least in those kept in darkness. The character of the neurosecretory material varied, too, in that heavy accumulations were commonly found in light-stimulated animals, while slender strands of neurosecretory material were typical of rats deprived of light.

It is, of course, not possible to judge by these means alone whether the neurosecretion passes into the capillaries of the median eminence and, if so, to what degree. It was, however, of great interest to note that the neurosecretory droplets became increasingly small, with increasing proximity to the blood vessels (shown best in Fig. 6). Beyond this point they could not be identified in these preparations with any degree of accuracy.

Study of the pars nervosa of 46 rats, both male and female (10 kept in darkness, 17 under continual lighting, and 19 under usual laboratory conditions) revealed differences in the amount of Gomori-positive material present. In animals living under normal laboratory conditions the neurosecretory material was most abundant, whereas in rats maintained under extreme excess or absence of light its amount was notably lessened (Figs. 9, 10, 11). Neurosecretory droplets were prominent in Herring bodies and along the axones in the control animals, while in the lighted rats these were scanty in both these sites. The decrease in neurosecretory material observed in the rats kept in darkness was largely confined to the axones, the Herring bodies usually being moderately large. It must be emphasized that within all three groups there was variation in response, yet when one group was compared with another, the changes noted were clearly evident. In Table 1 we have recorded our subjective estimates of the relative abundance of neurosecretory material in axones and Herring bodies on the basis of a scale of + to +++.

Examination of the vaginal smears revealed that while the rats housed



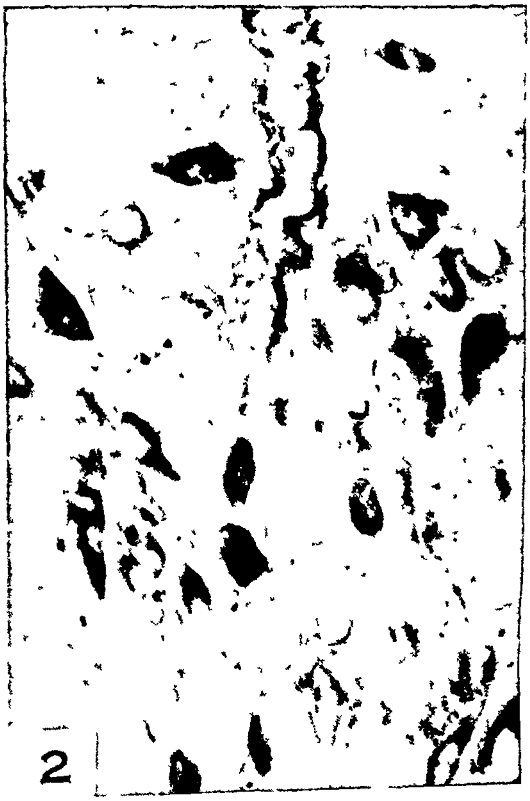
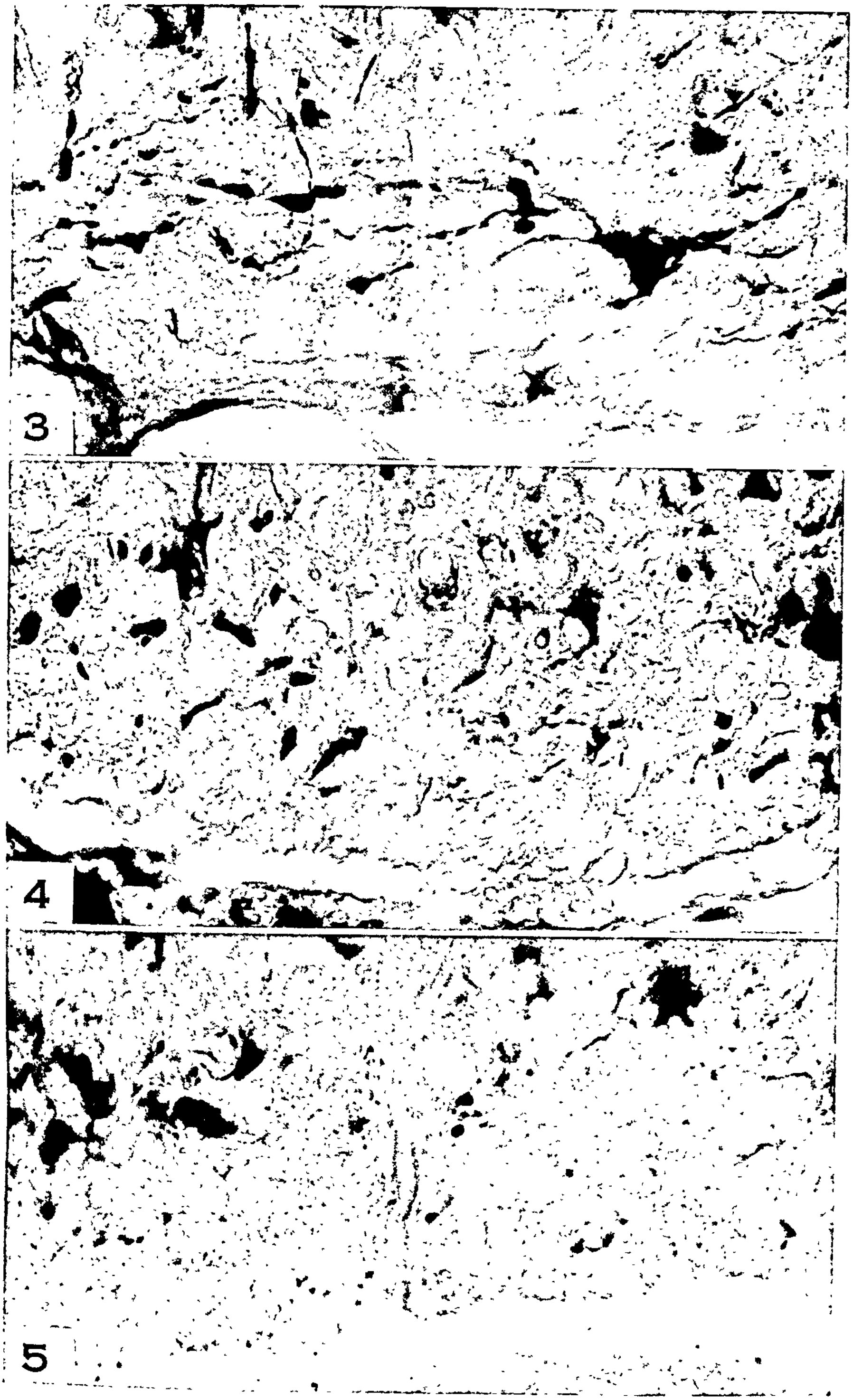
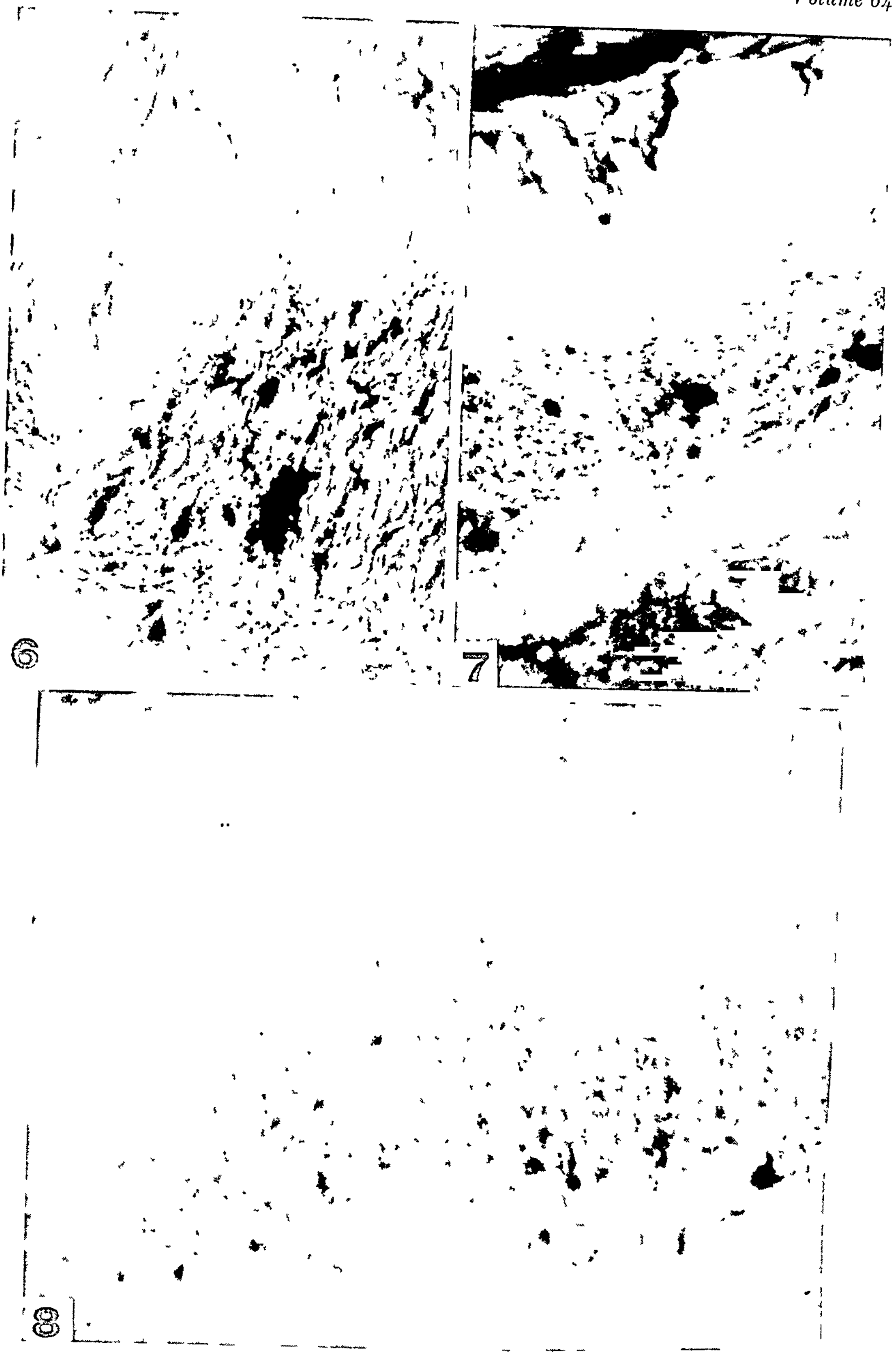


FIG. 1. Section through the supraoptic nucleus in a male rat continuously exposed to light showing the large size and active appearance of the neurosecretory cells characteristic of such animals. Aldehyde fuchsin and Halmi's counterstain. ×500. 10μ.

FIG. 2. Section through a supraoptic nucleus in a male rat kept in darkness showing some spindle cells and other neurosecretory cells typical of such animals. Aldehyde fuchsin and Halmi's counterstain. ×500. 10 μ.



Figs. 3–5. Sections through the hypothalamo-hypophysial tract ventral to the supra-optic nucleus in male rats kept in the light, under usual laboratory conditions and in darkness respectively. Aldehyde fuchsin and Halmi's counterstain. ×500. 10μ.



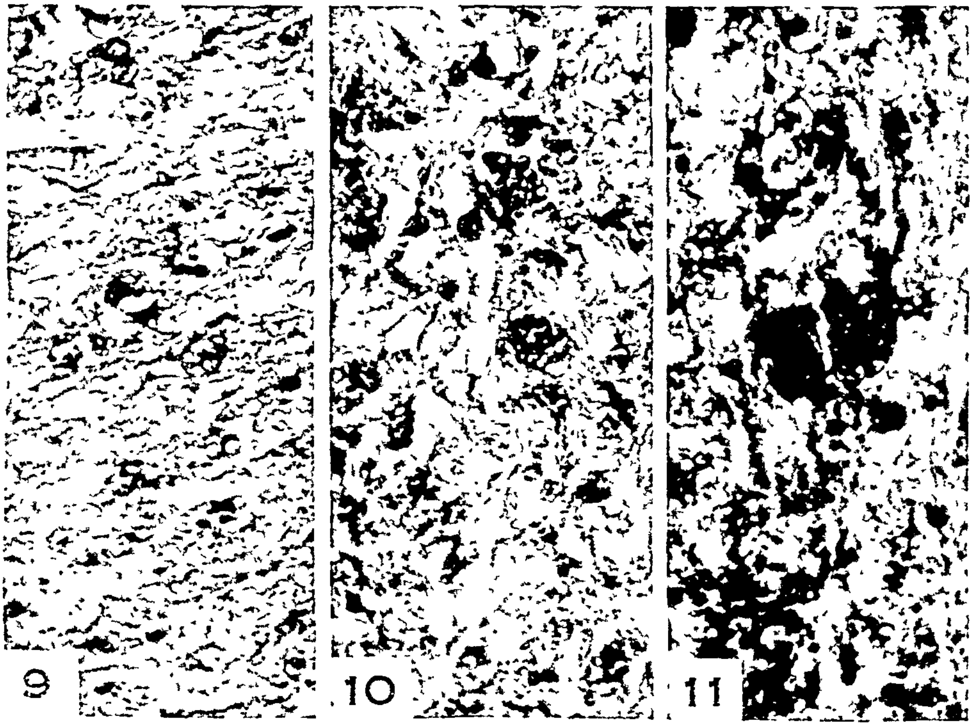
FIGS. 6–8. Sections through the hypothalamo-hypophysial tract in the median eminence in male rats kept in light, under usual laboratory conditions and in darkness respectively. Note the axones and associated Gomori positive material near the capillaries in Fig. 6. Aldehyde fuchsin and Halmi's counterstain. ×500. 10 μ.

under usual laboratory conditions were cycling regularly (showing the 4-day cycle typical of this strain), those in continuous light were cycling at intervals of approximately 7 days due to lengthened periods of estrus. Smears were not taken on the animals housed in darkness, but one of us has previously reported (3) that under similar conditions such rats cycle irregularly, exhibiting many metestrus smears.

Organ and body weights are presented in Table 2 and are in agreement with previous work in reflecting the gonad-stimulating action of added lighting. The appearance of the ovaries, too, was in line with earlier findings. Those of continuously lighted females, though not devoid of corpora lutea, had conspicuously large follicles, whereas the ovaries of the females housed in darkness were generally small, containing few well developed corpora lutea or follicles.

DISCUSSION

The observations reported here support the view that a neurosecretory link may be involved in the stimulation of the pituitary by light. Of especial interest is the fact that the supraoptic nuclei appear to be affected by changes in optic stimulation, while the paraventricular nuclei are not These two pair of nuclei are represented in the amphibia by the single N



FIGS. 9, 10 and 11. Sections through the pars nervosa of male rats exposed to altered light conditions for eight weeks. (9) Rats in continous light. (10) in constant darkness, (11) under control conditions. Note the differences in the amount of neurosecretory material in the Herring bodies and along the axones. Aldehyde fuchsin. ×600. 3μ

TABLE 1. RELATIVE AMOUNTS OF NEUROSECRETORY MATERIAL IN AXONES AND HERRING BODIES THROUGHOUT THE NEUROHYPOPHYSIS*

Control Rats			Rats in Constant Light			Rats in Constant Darkness		
	Axones	Herring bodies		Axones	Herring bodies		Axones	Herring bodies
				Females				
1	++	++	1	+	+	1	+	+
2	+++	+++	2	+	+	2	+	++
3	+++	+++	3	+	+	3	+	++
4	+++	+++	4	+	+	4	+	++
5	+++	+++	5	+	+			
6	+++	+++	6	+	+			
7	+++	+++	7	+	+			
8	+++	+++	8	++	+			
9	+++	+++	9	+	++			
10	++	++						
				Males				
1	+++	+++	1	+	+	1	++	++
2	+++	+++	2	+	+	2	+	+
3	+++	+++	3	+	++	3	+	+
4	+++	+++	4	+	+	4	+	+
5	++	++	5	+	+	5	+	++
6	+++	+++	6	+	+	6	++	++
7	++	++	7	+	+			
8	++	++	8	+	+			
9	++	+++						
19			17			10		Totals

* + Neurosecretory material scarce.
++ Neurosecretory material moderate.
+++ Neurosecretory material abundant.

praeopticus and both seem to be important in the production of posterior lobe hormones. It has been rather generally assumed that these nuclei perform common functions and respond similarly to experimental procedures. However, such a view is not consistent with the findings of Castor *et al.* (13) or with the extensive work of Olivecrona (14), whose studies point to the paraventricular nuclei as the primary source of oxytocin, and to the supraoptic nuclei as possibly the main source of vasopressin. Our

TABLE 2. BODY AND ORGAN WEIGHTS OF YOUNG MALE AND FEMALE RATS KEPT IN CONSTANT LIGHT, TOTAL DARKNESS, OR UNDER NORMAL CONDITIONS FOR EIGHT WEEKS

Treatment	No. of animals	Body wt. avg., gm.	Ovary avg. wt., mg.	Uterus avg. wt., mg.	Adrenal wt. avg., mg.
Light	15	184.8	68.4	342	47.7
Control	13	189.7	64.9	300	46.9
Dark	10	181.0	41.3	190	42.8
			Testes avg. wt., gm.	Sem. ves. avg. wt., mg.	
Light	18	223	2.96	375	30.8
Control	11	227	2.61	266	34.6
Dark	10	256	2.76	280	31.1

data likewise suggest some dissimilarity in the function of the supraoptic and paraventricular nuclei.

It is not possible on the basis of the present study either to draw conclusions concerning the mode of action of light on the supraoptic nuclei or to evaluate the functional significance, if any, of the morphological changes noted. The increased neurosecretory activity may have been induced directly by continuous optic stimulation in which event it may be related to the well-known light-induced changes in anterior pituitary secretion. The most evident shift in the anterior pituitary hormone balance brought about by constant lighting is the increased secretion and release of FSH and a tendency for the secretion of LH to be suppressed (3). The thyroids and adrenals of the light-exposed rats used in this study appeared to be functioning at normal levels as judged by weight and cytological appearance. But, it is noteworthy that rats subjected to sudden intense light respond by releasing an increased amount of ACTH (15). There is also evidence that under the influence of continuous light TSH-release is reduced in some rodents (16, 17). Thus, under appropriate conditions, the secretion of four of the anterior lobe hormones (FSH, LH, TSH, and ACTH) seems to be affected by light. It remains to be determined whether changes in the neurosecretory activity of higher centers, as noted here, can be related to alterations in anterior lobe functions.

The abundance of neurosecretory substance in the supraoptic nuclei, combined with the relative paucity of such material in the neural lobe in the rats exposed to light, suggests an increased release of neurohypophysial hormones. These circumstances, coupled with concomitant changes in gonadal activity, point again to the possibility that posterior lobe hormones may play a role in anterior pituitary physiology. Mirsky *et al.* (18), McCann and Brobeck (19), Martini and Morpurgo (20), and McCann (21) have advanced evidence in support of the view that neurohypophysial hormones are involved in ACTH secretion. The evidence, reviewed by Sayers, Redgate and Royce (22), is as yet inconclusive. Recently, Benson and Folley (23) have found that oxytocin injected into lactating rats increases LTH secretion, and Desclin (24) and Stutinsky (25) have induced decidua in animals injected with oxytocin. At the present time, however, there is no clear explanation of the role, if any, of the neurohypophysis in the regulation of the anterior pituitary.

There is the further possibility that the increased activity of the supraoptic nuclei of the rats housed in light may be an indirect response. Cavallera *et al.* (26) have reported that estrogen has a diuretic action in the rat, and De Groot (27) has suggested that the increased neurosecretory material which he found in the pars nervosa of some of his spayed rats may have been caused by the absence of this action of estrogen. Any diuretic action which estrogen may have had in the rats exposed to light as reported here was limited, for in no instance did the depletion of neurosecretory material approach that which follows severe dehydration as described by Leveque and Scharrer (28). The further observation by De Groot that some of his dehydrated rats continued to cycle although their posterior pituitaries were depleted of neurosecretory material is of interest. It suggests that cyclic reproductive activity can occur in the virtual absence of Gomori-positive material in the pars nervosa.

The "inactive" neurosecretory cells found in the rats housed in darkness were so classified in part because of their cytological features, but also because Hild and Zetler (29) have shown that neurosecretory cell bodies become filled with Gomori-positive material only when the cells have filled their "storage depots."

In view of the work reported here, assays of the posterior pituitary of light- and dark-treated animals might prove of interest. Furthermore, since previous work has shown that changes in anterior pituitary structure (30) and gonadotrophic content (3) are particularly marked in rats subjected to altered light conditions for the period used in this experiment, cytological studies of both the hypothalamus and the anterior pituitary in animals exposed for graded periods of time might be rewarding.

Acknowledgments

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ADDENDUM

Since the submission of this paper, Oksche *et al.* have reported changes in neurosecretion in the sparrow in response to light. (*Anat. Rec.* **130:** 433 abst. 1958).

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OBSERVATIONS ON THE PRODUCTION OF GOITER IN RATS WITH PROPYLTHIOURACIL AND ON GOITER PREVENTION[1]

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ABSTRACT

Rats fed propylthiouracil and injected with sodium-*l*-thyroxine (1.0 to 2.5 μg. per rat per day) developed goiter in the presence of normal concentrations of plasma protein-bound iodine. At all dose levels of thyroxine, rats fed low residue diets had greater serum bound iodine concentrations than animals fed commercial laboratory chow. Since goiter was found in presence of normal or high bound iodine concentrations, this may mean (a) goiter can develop despite low plasma levels of thyrotropin under these conditions or (b) plasma protein-bound iodine may have an abnormal composition or influence in these types of experiments. A method is presented for determination of plasma protein-bound iodine by isotope dilution.

DEMPSEY and Astwood (1) introduced the goiter-prevention method for estimating the daily rate of thyroid hormone secretion and presented as reasonable the statement: "The amount of thyroxine necessary to maintain normal thyroid weight in thiouracil-treated animals is a valid index of the rate of secretion of thyroid hormone." This has been widely accepted. They also stated: "In this instance, the balance is considered to be between the degree of pituitary inhibition exerted by the thyroid hormone and the quantity of thyroid secretion under the stimulus of thyrotropic hormone. According to this concept, then, an index of the degree of thyroid stimulation is provided by the size of the gland, which may be expected to vary directly with the rate of secretion of the thyrotropic hormone and inversely with the concentration of the circulating thyroid hormone." Since that time the estimation of circulating thyroid hormone, as protein-bound iodine, has become simpler, although the quantitation of thyrotropin in plasma remains difficult. However, the assumptions of Dempsey and Astwood have appeared so altogether reasonable that meas-

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urement has been neglected even in regard to "the concentration of the circulating thyroid hormone."

In the course of studies on the effect of high and low residue diets on the rate of fecal excretion of thyroxine we were surprised to gather data indicating that rats fed propylthiouracil and injected with sodium-*l*-thyroxine in graded dosage developed goiters unless the plasma protein-bound iodine concentration was raised to levels considerably above normal.

METHODS

The method of Dempsey and Astwood (1) was used to determine the amount of sodium-*l*-thyroxine injected subcutaneously daily which prevented goiter in rats fed diets containing propylthiouracil in 0.03% concentration. The thyroxine was dissolved in a slightly alkaline isotonic saline solution. The doses ranged from 1 μg. to 5 μg. per rat per day. The temperature of the animal room was 70 to 75° F.

Diet A consisted of gluten flour, 300 gm.; vitamin-free casein, 110 gm.; corn oil, 80 gm.; entire wheat flour, 100 gm.; sucrose, 370 gm.; salt mixture, 50 gm. The salt mixture contained the following: calcium carbonate, 300 gm.; potassium phosphate, dibasic, 150 gm.; magnesium sulfate, 50 gm.; sodium chloride, 500 gm.; ferrous sulfate, 1.0 gm.; copper sulfate, 0.05 gm.; manganese sulfate, 0.05 gm.; cobalt chloride, 0.05 gm.; sodium molybdate, 0.05 gm.; and zinc sulfate, 0.05 gm. A mixture of water-soluble vitamins was added. The iodine content was about 0.05 μg. per gm. of diet.

Diet B contained: corn starch, 270 gm.; sucrose, 270 gm.; casein, 260 gm.; corn oil, 80 gm.; salt mixture, 50 gm.; vitamin mixture, 50 gm.; and sodium iodide was added to make the iodine content 0.2 μg./gm. diet.

The third diet was Purina mink chow,[3] a high residue diet (2).

An experiment was made in which thyroxine was tagged initially with 30 μc. I^{131}-labelled thyroxine per 100 ml. of a solution containing 2.0 mg. of sodium-*l*-thyroxine. The purpose was to determine the plasma concentration of bound iodine by isotopic dilution, the method being similar to that reported for iodine metabolism studies (3). This provided an independent method for determining protein-bound iodine concentrations in plasma, based on the assumption of a state of equilibrium in which the specific activity of the plasma thyroxine was the same as that of the I^{131}-labelled stock solution of sodium-*l*-thyroxine.

Propylthiouracil and thyroxine were given for 10 to 14 days. The rats were anesthetized with ether 24 hours after the last injection of thyroxine. Blood was withdrawn from the inferior vena cava for determination[4] of protein-bound iodine by the method of Zak *et al.* (4) as modified by Astwood *et al.* (5). The thyroid glands were excised and weighed on a torsion balance.

RESULTS

Tables 1 and 2 indicate that the goiter-preventive dose of sodium-*l*-thyroxine was between 2.5 and 5.0 μg. per rat per day. However, with each level of thyroxine dosage the feeding of the low residue Diet B resulted in plasma concentrations of protein-bound iodine 1.3 to 2 times greater than were found with Purina chow. With the exception of two rats taking Diet

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[3] Iodine analyses on Purina mink chow showed 0.4 μg./gm. when 0.2 to 0.5 gram was analyzed directly (5).

[4] The technical assistance of Rosemary Libby, Howard Libby and Shirley Miller is gratefully acknowledged.

TABLE 1

Average body weight (No. rats)	Thyroxine dose, μg./day	After two weeks of thyroxine	
		Thyroid, mg./100 gm. body weight	Plasma bound iodine, μg./100 ml.
Diet A plus 0.03% propylthiouracil			
177 (Propylthiouracil not given, 5)	0	6.8 ±1.23*	5.5 ±0.35
161 (5)	0	28.2 ±10.3	2.5 ±0.95
149 (4)	1.3	20.2 ±2.2	5.2 ±0.6
149 (3)	2.5	17.0 ±2.5	7.5 ±0.4
148 (4)	5.0	8.4 ±3.3	11.1 ±2.2
Diet B plus 0.03% propylthiouracil			
162 (3)	1.3	17.0 ±3.8	7.1 ±0.6
160 (3)	2.5	14.6 ±4.6	9.3 ±1.2
166 (3)	5.0	5.3 ±0.8	16.8 ±2.6
Diet C (Purina) plus 0.03% propylthiouracil			
161.2 (Propylthiouracil not given, 10)	0	5.1 ±1.0	5.1 ±1.32
179 (8)	0	22.3 ±5.5	2.9 ±0.68
134 (7)	1.3	16.0 ±3.0	5.4 ±0.8
143 (6)	2.5	15.0 ±2.5	6.9 ±1.6
142 (4)	5.0	6.7 ±1.3	8.8 ±2.7

* Standard error of the mean.

B (Table 2) the rats injected with less than 5 μg. thyroxine per day had goiters despite concentrations of protein-bound iodine above the levels found in the untreated rats.

In the experiments recorded in Table 2 the concentration of protein-bound iodine was determined isotopically. It was considered that, after 10

TABLE 2

Body weight in grams (No. rats)	Thyroxine, μg./day	Thyroid, mg./100 gm. body weight	Plasma bound iodine,* μg./100 ml.
Diet B plus 0.03% propylthiouracil for 10 days			
131 (2)	1.2	15.0	5.1
134 (2)	2.5	9.0	10.2
134 (2)	5.0	8.0	16.5
Diet C (Purina) plus 0.03% propylthiouracil for 10 days			
139 (2)	1.2	19.0	2.8
134 (2)	2.5	12.0	4.8
153 (2)	5.0	6.0	7.9

* Plasma thyroxine/100 ml. = (PBI^{131} counts/min./100 ml. plasma) ÷ (counts/min./μg. thyroxine in solution of radiothyroxine at time of radioactive determination)

Bound iodine is 63.6% of calculated sodium thyroxine.

days of administration of propylthiouracil and injections of radio-thyroxine, the plasma concentration of protein-bound iodine would be largely due to injected thyroxine. The specific activity (I^{131}/μg. thyroxine) in plasma would approach that of the solution itself. Therefore, the determination of I^{131} in the trichloracetic acid precipitate of plasma would indicate a minimum value for the concentration of protein-bound iodine. The table indicates that rats which received 1.2 μg. to 2.5 μg. sodium-*l*-thyroxine per day developed goiters from propylthiouracil when the plasma concentration of protein-bound iodine was normal. In Table 2 the only rats which had lowered plasma concentrations of protein-bound iodine were those fed Purina chow and injected with 1.2 μg. *l*-thyroxine per day. Feeding the low residue diet to rats given daily injections of thyroxine appears to have resulted in plasma concentrations of protein-bound iodine twice those found when the high residue Purina chow diet was fed.

DISCUSSION

This study shows that goiter prevention in propylthiouracil-treated rats is only achieved through daily injections of sodium-*l*-thyroxine in amounts which elevate the plasma concentration of protein-bound iodine to values 2 to 3 times normal. This clearly raises the possibility that the goiter-prevention method of Dempsey and Astwood (1) gives an erroneously high value for the daily secretion rate of thyroxine by the thyroid gland. Taurog and Chaikoff (6), using a method involving specific activities of diiodotyrosine and thyroxine in the thyroid gland, calculated a thyroxine secretion rate of 1.5 μg./100 gm. body weight per day, in contrast to the 5.2 μg. figure of Dempsey and Astwood. These two groups used different diets.

If, under the conditions of these experiments, protein-bound iodine determinations provide a measure of circulating thyroid hormone, it is likely that goiter has developed under conditions in which thyrotropin production is normal. Goldberg *et al.* (7) have reviewed briefly the point of view that thyroid-pituitary relationships are not fully explained by a simple feedback mechanism, and the present study provides further support for this contention. It was first suggested by Albert *et al.* (8) and confirmed by Halmi and Spirtos (9) that the effects of thyrotropin on thyroid cell height were enhanced by antithyroid drugs. Rawson (10) has taken the position that antithyroid drugs cause goiter through two mechanisms: by enhancing thyrotropin output in response to hypothyroidism and by potentiating the effect of thyrotropin. This formulation allows the inference that goiter may occur despite some suppression in thyrotropin production, and this study provides a basis for exploring this possibility. Other manipulations which deplete thyroid iodine stores have in common with propylthiouracil the augmentation of iodide concentrating activity (11, 12). It seems likely that goitrogenesis due to feeding of propylthiouracil to intact rats having high protein-bound iodine concentrations, as observed in this study, is due directly to an increased thyroid sensitivity to thyrotropin.

Among possible objections to attaching significance to these findings is the non-specificity of protein-bound iodine in plasma as a measure of circulating thyroid hormone. With the low iodine diets this objection seems less significant than in the experiments in which dietary iodine was higher. Objection also may be held on the score that normally triiodothyronine is also a component of the protein-bound iodine. However, Gross (13) has found that the concentration of triiodothyronine in plasma is probably very much less than that of thyroxine and, in addition, Gross and Leblond (14) found Compound No. 1, subsequently shown to be triiodothyronine (15), in the plasma of thyroidectomized rats injected with thyroxine.

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STUDIES IN THE ACTIVATION AND INHIBITION OF ADRENOCORTICOTROPHIN SECRETION

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ABSTRACT

Comparisons have been made in the depletion of adrenal ascorbic acid which resulted from cold, hypertonic saline and bell-ringing, in normal, neurohypophysectomized and reserpine injected male rats. The effects upon ACTH/AAF release of the stimulation of neurohypophysial hormone release, or its injection, were recorded in normal animals and neurohypophysectomized animals subjected to cold stress. Also, the effects of neurohypophysectomy and reserpine injection upon the adrenal weights of normal and adrenal-enucleate-regenerate animals were recorded.

It was concluded that:

1. The liberation of AAF by the adenohypophysis seems to necessitate the concomitant release of neurohypophysial hormone or something associated with it and it is suggested that the release of ADH by the neurohypophysis results in an "obligatory" release of AAF.
2. The neurohypophysis is involved in the release of ACTH/AAF in response to systemic stresses but not to emotional ones. It is suggested that separate centres within the hypothalamus control, via different pathways, the secretion of AAF by the adenohypophysis.
3. The hypertrophy of the adrenals which results from neurohypophysectomy, and the regeneration of the adrenals after enucleation, are discussed and related to the results of other workers. It is suggested that enucleated adrenals regenerate under the influence of a basal secretion of ACTH/AWF, the control of which probably does not involve hypothalamic centres and that the release of a hypertrophy AWF involves a hypothalamic centre the stimulation of which depends upon the capacity of the centre/s which stimulate/s AAF release, to meet body requirements.

THE mechanisms involved in the release of ACTH from the pituitary are, to say the least, complex. ACTH itself appears to have a dual function in that it maintains adrenal weight and stimulates an increase in the release of steroids from the adrenal cortex (1) and these two functions are obviously related to one another since adrenocortical volume varies according to the demands made upon its productive capacity. In addition, it has become customary to refer to two categories of stress, both of which result in an increased secretion of ACTH (2). These are, neurotropic or emotional stress which activates the anterior lobe of the pituitary via the central nervous system and hypothalamus, and metabolic or systemic

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stress which generally results in a more direct action of blood-bourne agents upon the anterior lobe but which may also involve the central nervous system (3, 4, 5 and 6). Since de Groot and Harris (7) postulated a chemical transmitter passing from the hypothalamic area via the hypophysial portal vessels to stimulate the anterior lobe, many workers have attempted to relate this unknown agent to the Gomori-positive neurosecretory material demonstrated in the hypothalamic nuclei and known to be intimately related to the functioning of the neurohypophysis(8).

The present experiments were designed in order to attempt to relate the function of the neurohypophysis to the release of ACTH by the adenohypophysis. They involve the stimulation or inhibition of one or both of these organs with the above mentioned findings in mind, and the extent to which the results shed light upon the control of ACTH secretion is discussed.

MATERIAL AND METHODS

Male albino rats of the Wistar strain were used in all experiments. They were kept at a temperature of about 70° F and fed on commercial rat cake and water *ad lib.* The percentage depletion of adrenal ascorbic acid resulting from any stress and the percentage adrenal weight at the end of the experimental period were used as criteria for comparing the amount of ACTH (Ascorbic acid factor, AAF, and Adrenal weight factor, AWF, respectively) liberated by the pituitary under the various experimental conditions. The experimental groups were divided up as follows:

Blockage of ACTH (AAF) secretion

Normal control animals subjected to stress

a. Cold. Those rats to be subjected to stress were placed in the cold room for 1 hour at 30° F and sacrified 1 hour after the stress.
b. Bell ringing. Animals to be stressed were placed in a separate room and subjected to the ringing of a loud electric bell for a period of 10 minutes. They were sacrificed half an hour after the stress.
c. Hypertonic saline. The animals to be stressed received an infusion of 5 ml. of warm 308 mM saline over a period of 3 minutes and were sacrified an hour later. Earlier experiments designed to induce the secretion of ACTH by the administration of hypertonic saline solutions via stomach tube, proved to be abortive, since the act of inserting the stomach tube provided an emotional stress which was sufficient to mask the effect of the saline. Thus in this and all other experiments involving saline infusion, a polythene canula was inserted into the jugular of both unstressed and stressed animals 48 hours before the infusion and the unstressed animals were killed 1 hour after a mock infusion.

Neurohypophysectomized animals subjected to stress

The parapharyngeal approach was used in the removal of the posterior lobe of the pituitary and the anterior lobe was only very slightly damaged. Normal increase in body weight and adrenal hypertrophy, which follow this operation, confirm that the anterior lobe continues to function. All stress experiments were performed 10 days after the operation, during which period the rats drank 4-5 times normal quantities of water. The depletion of adrenal ascorbic acid, which resulted from the subjection of these rats to the stresses of cold, bell-ringing and hypertonic saline, was compared with the corresponding figure in the normal control animals.

Animals injected with reserpine and subjected to stress

All rats due to be stressed received a subcutaneous injection of 1 mg./kg. body weight of resperpine the day before the experiment and the same dose was given half an hour before stressing. The experiments were performed simultaneously with those on the normal animals and the same three types of stress were imposed. Using the same unstressed controls the depletion of adrenal ascorbic acid was again compared with that obtained in the normal animals.

Stimulation of ACTH (AAF) secretion

In considering the relationship between posterior and anterior lobe secretion, the following experiments were designed in order to stimulate or to substitute for posterior lobe secretions and to relate the resulting effects to ACTH secretion:

Normal animals

a. Intravenous infusion of Tyrode's Ringer solution. 5 ml. of warm Ringer solution were infused, over a period of 3 minutes, into rats which had been canulated 48 hours before and the rats killed 1 hour later.
b. Intravenous infusion of hypertonic saline. The stressed rats received 5 ml. of 308 mM saline (as in c above).
c. Intravenous infusion of Ringer plus Pitressin. 0.3 u. of Pitressin (Parke, Davis) was added to the Ringer solution and the whole made up to 5 ml.
d. Intravenous infusion of Ringer plus Pitocin. 0.3 U. of Pitocin (Parke, Davis) was added to the Ringer solution and the whole made up to 5 ml.
e. Intravenous infusion of Ringer plus nicotine. Two mg./kg. body weight of nicotine were added to the Ringer solution and the whole made up to 5 ml.

Neurohypophysectomized animals subjected to cold stress

The procedure followed that for neurohypophysectomized animals subjected to cold stress (see above) except that the stressed animals received a subcutaneous injection of one of the following substances half an hour before they were placed in the cold room.

a. 0.3 u. of Pitressin
b. 0.3 u. of Pitocin
c. 2mg./kg. body weight of nicotine.

Animals injected with reserpine and subjected to the stress of bell-ringing

The procedure followed that for resperpine injected animals subjected to bell-ringing (see above) except that a second group of stressed animals received 0.3 u. of Pitressin together with 1 mg./kg. of resperine half an hour before stressing.

Hypophysectomized control animals

These animals were sacrificed 1 hour after they received a subcutaneous injection of 0.3 u. of Pitressin plus 0.3 u. of Pitocin.

The influence of neurohypophysectomy and reserpine upon ACTH/AWF secretion

In the course of this and previous work it was found that neurohypophysectomy resulted in a marked increase in adrenal weight, presumably due to an increased secretion of ACTH/AWF. It is also generally accepted that the secretion of this hormone is increased after adrenal enucleation (9) as it is after adrenalectomy (10) and prolonged stress (11). The adrenal weights of the animals subjected to the following conditions were recorded in an attempt to relate the influences of neurohypophysectomy and reserpine to adrenal weight.

a. Neurohypophysectomized normal animals. Adrenal weights were taken 10 days after the operation.

b. Neurohypophysectomized adrenal-enucleate-regenerates. Neurohypophysectomy was performed 1 day before adrenal enucleation. Both adrenals were enucleated in this and all other enucleate groups mentioned and all adrenals were allowed to regenerate for 7 days.

c. Reserpine injection of adrenal-enucleate-regenerates. Reserpine (1 mg./kg.) was injected daily throughout the 7 day period of regeneration.

d. Reserpine injection of adrenal-enucleate-regenerates stressed by bell ringing. An electric bell was placed immediately above the cages and set to ring for 55 seconds every hour throughout the 7 days of regeneration. Reserpine (1 mg./kg.) was injected daily throughout this 7 day period.

e. Reserpine injection of neurohypophysectomized animals. Reserpine (1 mg./kg.) was injected daily throughout the 10 day experimental period.

RESULTS

Blockage of ACTH (AAF) secretion

The effect of the removal of the posterior lobe of the pituitary upon the response of the anterior lobe to the stresses of cold, bell ringing and hypertonic saline are shown in Table 1. The figures for the percentage depletion of adrenal ascorbic acid indicate that this operation reduces the release of ACTH/AAF by the adenohypophysis in response to the stresses of cold and the intravenous infusion of hypertonic saline but not to the stress of bell ringing. The reverse is true after reserpine injection since the response to both cold stress and hypertonic saline infusion remained normal whilst the response to bell ringing was abolished altogether.

Stimulation of ACTH (AAF) secretion

The effects of the attempted stimulation of ACTH secretion in normal, neurohypophysectomized and hypophysectomized animals are shown in Table 2. In the normal animals the infusion of 5 ml. of Tyrode's Ringer solution resulted in a 7.6% depletion of adrenal ascorbic acid (P= >0.3 <0.4). However, when 308 mM saline was substituted for

TABLE 1. THE EFFECT OF COLD (1 HOUR AT 30° F), BELL RINGING (10 MINUTES) AND INTRAVENOUS 308 MM SALINE UPON THE PERCENTAGE ASCORBIC ACID CONTENT OF THE ADRENALS IN NORMAL, NEUROHYPOPHYSECTOMIZED AND RESERPINE INJECTED (1 MG./KG. BODY WT.) RATS

Category of rat	Treatment of stressed animals	Ascorbic acid mg./100 gm. of adrenal tissue ± S.E.		% depletion on stress	'P' value
		Unstressed	Stressed		
Normal	Cold	423.7 ± 23.7 (8)	317.2 ± 13.7 (9)	25.1	<0.01
	Bell ringing	408.5 ± 13.1 (6)	338.5 ± 13.2 (6)	17.1	<0.01
	308 mM saline	316.1 ± 16.8 (10)	235.4 ± 10.6 (9)	25.5	<0.01
Neurohypophysectomized	Cold	369.8 ± 20.1 (9)	365.6 ± 18.2 (9)	1.1	>0.8
	Bell ringing	398.0 ± 17.7 (7)	323.1 ± 18.7 (8)	18.8	0.01
	308 mM saline	322.8 ± 19.0 (7)	306.1 ± 13.0 (7)	5.1	<0.5 >0.4
Normal	Injected with 1 mg./kg. reserpine and subjected to				
	Cold	423.7 ± 23.7 (8)	317.0 ± 14.5 (8)	25.1	<0.01
	Bell ringing	408.5 ± 13.1 (6)	426.6 ± 13.0 (6)	4.4 incr.	—
	308 mM Saline	316.1 ± 16.8 (10)	247.4 ± 14.5 (7)	21.7	<0.01

( ) No. of animals.

TABLE 2. THE EFFECT OF NEUROHYPOPHYSIAL STIMULATION AND THE ADMINISTRATION OF NEUROHYPOPHYSIAL HORMONES (PARKE, DAVIS PITRESSIN AND PITOCIN) UPON THE ASCORBIC ACID CONTENT OF THE ADRENALS OF VARIOUS CATEGORIES OF RATS

Category of rat	Treatment of stressed animals	Ascorbic acid—mg./100 gm. of adrenal tissue ± S.E.		% depletion on stress	'P' value
		Unstressed	Stressed		
Normal	*Infusion of* Ringer 308 mM saline Ringer+0.3 u. Pitressin Ringer+0.3 u. Pitocin Ringer+2 mg./kg. Nicotine	305.7 ± 15.2 (8) 316.0 ± 11.6 (12) 347.2 ± 14.7 (9) 299.4 ± 15.2 (9)	282.5 ± 15.4 (8) 251.0 ± 13.2 (10) 263.7 ± 19.7 (7) 260.7 ± 20.7 (7) 223.8 ± 6.7 (8)	7.6 20.6 24.0 25.0 25.2	>0.3 <0.4 <0.01 <0.01 <0.01 <0.01
Neurohypophysectomized	*Cold Stress and injection of* 0.3 u. Pitressin 0.3 u. Pitocin 2 mg./kg. Nicotine	496.0 ± 26.4 (10) 516.0 ± 28.0 (9) 495.7 ± 26.0 (7)	375.6 ± [illegible].1 (9) 412.2 ± 27.0 (9) 401.1 ± 22.0 (7)	24.2 20.1 19.0	<0.01 <0.02 >0.01 <0.02 >0.01
Normal	*Stressed by bell ringing and injected with* 1 mg./kg. Reserpine 1 mg./kg. Reserpine plus 0.3 u. Pitressin	381.1 ± 22.2 (6)	376.0 ± 13.1 (7) 277.0 ± 13.9 (7)	1.3 27.3	>0.8 <0.01
Hypophysectomized	*Injection of* 0.3 u. Pitressin plus 0.3 u. Pitocin	367.5 ± 28.7 (8)	375.4 ± 17.1 (8)	—	

( ) No. of animals

Ringer solution or when Pitressin, Pitocin or nicotine were added to the Ringer solution, the depletion of adrenal ascorbic acid became significant—around the level of 24%.

The adrenal ascorbic acid content in the unstressed animals of groups which had been subjected to jugular canulation in preparation for infusion was lower than in non-canulated groups. This is probably due to the stressing effects of canulation and reflects an increased output of ACTH in these groups. The significance of this control level of adrenal ascorbic acid has been discussed previously (9).

In the neurohypophysectomized animals, where cold stress had resulted in the insignificant depletion of adrenal ascorbic acid of 1.1% (P= >0.8) the injection of Pitressin, Pitocin or nicotine, before the stress was applied, resulted in a significant depletion (around 21%) in each case.

Also, in normal animals which showed a negligible depletion of adrenal ascorbic acid in response to bell ringing, when pre-treated with reserpine, the addition of 0.3 u. of Pitressin to the injected reserpine resulted in a normal depletion of 27.3%.

Hypophysectomized animals acted as controls for the above groups and in these animals the injection of a solution containing both Pitressin and Pitocin produced no depletion of adrenal ascorbic acid.

The influence of Neurohypophysectomy and reserpine upon ACTH(AWF) secretion

The final percentage adrenal weights of the various categories of rats used in these experiments are shown in Table 3. After 10 days, during which time they drank 4 to 5 times normal quantities of water, neurohypophysectomized animals showed a percentage adrenal weight of 25.23 mg. as against 15.61 mg. in the normal animal. The daily injection of 1 mg./kg. of reserpine did not prevent this increase in adrenal size. Bilaterally enucleated adrenals in the normal animal regenerated to a weight of 11.2 mg.% in 7 days and similar animals which had been previously neurohypophysectomized, although drinking similar quantities of liquid (Figure 1), showed a regeneration weight of 21.9 mg. %. Lastly, the regenerated adrenals of animals which were subjected to bell ringing throughout the period of regeneration were heavier (17.35 mg. %) than those of the control animals (10.38 mg. %) and the daily injection of 1 mg./kg. of reserpine, which had no significant effect upon the regeneration of the control adrenals (12.03 mg. %), did prevent the hypertrophy which had resulted from bell ringing (13.0 mg. %).

TABLE 3. THE INFLUENCE OF NEUROHYPOPHYSECTOMY AND RESERPINE UPON ADRENAL WEIGHT

Treatment	No. of rats	Final average body weight, gm. ±S.E.	Adrenal weight—mg./100 gm. body ±S.E.	
			Controls	Neurohypophysectomized
Normal	39	224.6 ±2.9	15.61 ±0.78	—
	19	210.5 ±2.5	—	25.23 ±1.02
Adrenal-enucleate-regenerate	9	173.6 ±3.6	11.2 ±0.736	—
	6	133.3 ±3.5	—	21.9 ±2.1
			Controls	Reserpine injected, 1 mg./kg. daily
Adrenal-enucleate-regenerate	17	141.3 ±2.1	10.38 ±0.47	—
	10	120.6 ±4.7	—	12.03 ±1.12
Adrenal-enucleate-regenerate stressed by bell ringing	7	142.1 ±4.5	17.35 ±1.24	—
	7	133.1 ±4.5	—	13.0 ±0.73
Neurohypophysectomized	6	153.0 ±8.4	27.9 ±3.28	—
	9	175.5 ±7.6	—	33.9 ±2.6

DISCUSSION

The present findings on the relationship between hormones of the neurohypophysis and ACTH secretion are in agreement with those of Sobel, Levy, Marmorston and Rosenfeld (12), Martini and DePoli (13) and others. The administration of Pitressin or Pitocin induced a significant depletion of adrenal ascorbic acid in animals which had not otherwise shown such a response. Also, the induction of ADH release, by the administration of nicotine (see 14) and hypertonic saline (see 15), produced similar depletions of ascorbic acid in stressed animals which had shown only slight responses in the absence of these stimulating substances. That this ACTH release is not due to any necessity for additional corticosteroids at the kidney level was demonstrated, firstly by the finding that little ACTH was released when hypertonic saline was administered to neurohypophysectomized animals with an otherwise functional anterior lobe and secondly, by the finding that ACTH was not released when a Ringer load alone was administered, but that the addition of Pitressin, Pitocin or nicotine to the Ringer did cause a depletion of adrenal ascorbic acid. This latter reaction must be mediated via the pituitary since no response to Pitressin and Pitocin injection was obtained in hypophysectomized animals.

Thus the presence of posterior lobe hormones (or something associated

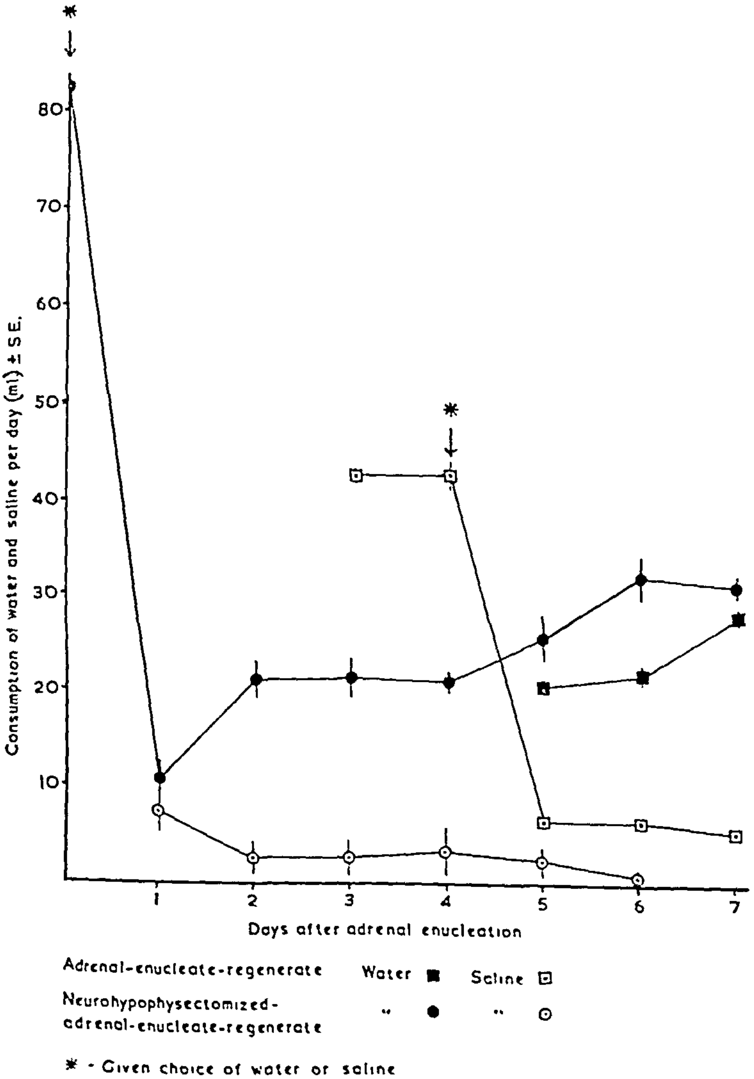

FIG. 1. Consumption of water and saline (0.9%) by adrenal-enucleate-regenerate and neurohypophysectomized-adrenal-enucleate-regenerate rats.

with them) in the circulation appears to be essential to ACTH release, and, moreover, it is suggested that the presence of these hormones at the level of the pituitary results in a direct "obligatory" release of ACTH. On the other hand, it would appear that mechanisms which are known to result in the release of ACTH also involve the concomitant release of ADH, as in emotional stress (16 & 17) or systemic stress (15). However, the above experiments suggest the existence of two distinct mechanisms of ACTH release. One of these mechanisms, which responds to emotional stress, may, at some unknown level, be stimulated by nicotine and can be blocked by reserpine. The other responds to systemic stresses such as cold and hypertonic saline and, unlike the former, necessitates the presence of an intact neurohypophysis. These findings agree generally with results of corresponding work on ADH release reported by Dingman (18), Dingman and Thorn (19) and Dingman and Despointes (20) who concluded that nicotine stimulated hypothalamic centres to release ADH in patients with diabetes insipidus, whereas hypertonic saline did not. They also found that, in normal subjects, the nicotine induced decrease in free water clearance was blocked by cortisone, hydrocortisone and prednisolone, whereas that to hypertonic saline and Pitressin was not.

Thus, although these two mechanisms of stress response have something in common—the release of posterior lobe hormone—the present experiments suggest the following conclusion. That emotional stresses involve the passage of neurosecretory material, possibly from nuclei in the anterior region of the hypothalamus, to the adenohypophysis, via the main portal vessels, so that neurohypophysectomy would not interfere with this mechanism. That systemic stresses involve the passage of such material, possibly from more posterior nuclei of the hypothalamus, to the adenohypophysis, via other portal vessels within posterior lobe tissue, so that this pathway would be servered by neurohypophysectomy. These suggestions are strongly supported by the work of Daniel and Prichard (21) who found that in the rat cautery of the hypophysial portal vessels resulted in necrosis of only the central part of the adenohypophysis and that the tissue which did not become necrosed was supplied by uncauterised portal vessels which drain part of the neural lobe.

The relationship between ACTH/AAF and ACTH/AWF secretions, if these can be considered separately, are obviously complex. It is known that, firstly, the weight of an animal's adrenals is, under normal circumstances, related to the weight (22), the surface area (23) and the sex (24 & 25) of the animal; secondly, that the removal of one adrenal results in the compensatory hypertrophy of the other (26 & 27); thirdly, that the demands for corticoids which result from stress bring about an increased secretion of ACTH/AAF and, depending upon the extent of this demand, adrenal hypertrophy (11). These and other findings (9), which attempt to relate the sex of the animal to the adrenal weight and its capacity to respond to stress, would suggest the existence of a balance to control the ratio of AAF/AWF liberated by the pituitary and which might be regulated by the degree of stimulation or inhibiton of separate but closely associated centres in the hypothalamus.

In the present experiments neurohypophysectomized normal animals, which did not release AAF in response to systemic stress, showed normal body weight increase and a considerable degree of adrenal hypertrophy even when reserpine, which had been shown to block the hypertrophy due to bell ringing, was injected daily. Also, the regeneration weight of enucleated adrenals, during a seven day period, was doubled when animals were previously neurohypophysectomized and this increase occurred in spite of a normal consumption of water. These findings agree with those of McCann and Brobeck (28) who showed that, following lesions of the supra-optico-hypophysial tract, adrenal hypertrophy resulted in cases with high diabetes insipidus. However, this hypertrophy resulted in some cases but not in others. Similarly, Greer and Erwin (29) found that adrenal atrophy resulted from destruction of the medium eminence in some cases, but in others there was no correlation between destruction of the supra-optic and paraventricular nuclei and the absence of amphenone-induced adrenal hypertrophy. They suggested, therefore, that Pitressin may not play a significant role in the control of corticotrophin secretion. If, as previously suggested, separates centres *are* responsible for the release of AAF and AWF, then the release of AAF might depend upon the degree of hypothalamic stimulation balanced against the titers of blood corticoids, and the release of AWF upon the degree of hypothalamic stimulation plus the capacity of AAF secretion to meet this balance. Adrenal atrophy, which follows corticosteroid administration, would, on this hypothesis, be due to the fact that AAF and hence AWF, were no longer required. Also, the presence of high blood corticoids together with adrenal hypertrophy, as is found in continuous stress, could be accounted for.

On this hypothesis too, the findings of McCann and Brobeck and Greer and Erwin could be explained. A median eminence lesion might destroy either the centre responsible for the stimulation of AAF release or that for AWF release or, if they are closely related, both, so that ambiguous results may be obtained. In connection with Greer and Erwin's suggestion that Pitressin may not play a significant role in the control of corticotrophin secretion, the present data would suggest that this hormone is associated with the release of AAF only, so that if the supra-optic and paraventricular nuclei are responsible for the release of AAF, then the destruction of these nuclei might *induce* adrenal hypertrophy. A similar reaction is suggested for the adrenal hypertrophy in neurohypophysectomized rats in which the release of AAF in response to systemic demands was blocked. This hypertrophy tended to be even greater when, in addition, the response to emotional stresses was blocked by reserpine.

In addition to the consideration of an ACTH factor responsible for adrenal hypertrophy the findings in adrenal-enucleate animals are of interest since they agree with those of Ganong and Hume (30) who suggest that the pituitary maintains a basal secretion of ACTH in the presence of lesions of the median eminence which abolish compensatory and stress-induced adrenal hypertrophy. It appears that the adrenals regenerate, under the influence of this basal secretion, to the percentage weight of 10–11 mg. This secretion is not blocked by reserpine, but just as Ganong and Hume prevented stress-induced adrenal hypertrophy by hypothalamic lesions, so I blocked this hypertrophy by the injection of reserpine. Schneider, Holden, Rinehart and Moore (31) showed that reserpine probably blocks afferent impulses and does not depress centres within the hypothalamus, so that although the mechanism whereby this hypertrophy was blocked probably differed in the two cases, a distinction between the mechanisms involved in the release of the basal secretion on the one hand and the factor responsible for adrenal hypertrophy on the other, is indicated.

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THE EFFECTS OF ATRIAL STRETCH ON ALDOSTERONE SECRETION[1]

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ABSTRACT

A study was carried out to determine whether the secretion rates of aldosterone and of hydrocortisone are subject to regulation by receptors located in the atria of the heart. In dogs, the right or left atrium was stretched with sutures which extended from the atrium to the thoracic wall. Adrenal venous blood was collected and analyzed for aldosterone and hydrocortisone. Following right atrial stretch the aldosterone secretion rate was significantly reduced. However, stretching the left atrium was without effect on the secretory rate of aldosterone. These findings strongly suggest that the secretion of aldosterone may be regulated in part by stretch receptors located in the right atrium.

SOME years ago J. P. Peters postulated that the secretion of electrolyte-active steroids is under the control of the volume of the vascular system (1). The theory has been widely discussed (2, 3). The demonstration that aldosterone is the principle electrolyte-active steroid of the adrenal cortex stimulated reinvestigation of this concept. Urinary aldosterone levels fall after water-loading and rise after loss of body water (4, 5, 6). Loss of blood has been shown to stimulate aldosterone secretion in the dog (7, 8). The well-documented increase in aldosterone secretion following sodium deprivation (9) has been thought by some workers to be mediated by contraction of body fluid compartments rather than by direct action of the sodium ion (6).

However, the physiological system whereby changes in body fluid volume mediate changes in steroid output has remained unknown. Receptors, afferent pathways and central integrative mechanisms have never been elucidated. Henry and his collaborators have demonstrated that distension of the left atrium in dogs induces a prompt diuresis and have proposed that atrial stretch receptors play a part in the homeostatic regulation of water balance (10). It was of considerable interest to determine whether a similar volume receptor mechanism might exist for the control of electrolyte homeostasis, mediated by the adrenal cortex. Unfortunately the reports of

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these workers do not shed light on the problem. In Henry's experiments the atria were distended for only a few minutes at a time. Significant changes in the rate of steroid secretion occur rather slowly (11). Following complete hypophysectomy the steroid secretion rate remains high for 15 minutes or longer, and then gradually declines, reaching the low rate characteristic of hypophysectomy only after approximately one hour. Steroid mediated changes in urinary electrolytes would not be anticipated in the short time periods studied by Henry and his coworkers. Further, the technique of placing balloons in the atria (which introduces the complication of reducing cardiac output) does not permit study of the effects of longer-term distension of the atrium. Accordingly, a simple technic was devised to stretch the atria without the introduction of balloons. The results indicate that the right atrium contains receptors which when subjected to stretch act to inhibit the secretion of aldosterone. It is postulated that this system may function to control steroid secretion in response to changes in the volume of blood in the right atrium.

METHODS

Eighteen male mongrel dogs weighting 15.5 to 26.5 kg. were divided among four experimental groups as follows: controls (4), open chest shams (4), open chest plus left atrial stretch (5), and open chest plus right atrial stretch (5). In each experiment the animal was anesthetized with sodium pentobarbital and an external jugular vein and a common carotid artery cannulated for subsequent infusions and mean arterial blood pressure determinations. In the four control experiments the left lumbo-adrenal vein was cannulated and adrenal venous blood was collected for a two hour period. In the other three groups of animals the chest was opened by a transverse incision in the left third or fourth intercostal space and alternating positive pressure artificial respiration was instituted. An incision was made in the pericardium ventral to the left phrenic nerve of sufficient length to expose either the right or left atrial appendage. The cut edges of the pericardium were elevated and secured to the chest wall.

For right atrial stretch, a suture was passed through the tip of the atrial appendage. Similar sutures were placed between the base of the appendage and the sulcus terminalis, one near the entrance of the superior vena cava and the second near the entrance of the inferior vena cava. Sufficient tension was applied to the sutures to visibly stretch the atrial wall in a circumferential manner, without changing the general orientation of the heart structures. The free ends of the sutures were then secured to the wound edges to maintain the tension.

For left atrial stretch, three sutures were also employed, one at the tip of the atrial appendage and the second and third near the openings of the pulmonary veins on opposite sides of the base of the appendage. In the sham-operated animals, sutures were placed without tension in the right atrium (2 dogs) and in the left (2 dogs). In each experiment the exposed portion of the heart was covered with gauze moistened with warmed saline.

One hour following stretch of the right or left atrium, or placing of sutures in the sham-operated groups, the left lumbo-adrenal vein was cannulated and adrenal venous blood collected for a two-hour period. Periodic rectal temperature and mean arterial blood pressure observations were made. The temperature was maintained between 35 and 40° C with heat lamps. Arterial blood pressure was supported when it fell below 80 mm. Hg by infusing 0.2% norepinephrine in 5% glucose. Heparin (100 mg.) was injected intravenously just prior to the cannulation of the adrenal vein to prevent clotting.

The adrenal venous effluent was collected in vessels at 0° C. Immediately following the collection period the sample was diluted with an equal volume of water and extracted with a volume of chloroform equal to that of the blood-water mixture or frozen for subsequent extraction. Aldosterone and hydrocortisone were isolated by paper chromatographic methods (12) and quantitated by absorption at 240 $m\mu$. in methanol. Evidence for the identity of aldosterone, which was isolated as the diacetate, was obtained by mixed chromatograms with authentic aldosterone diacetate, and by bioassay following hydrolysis (acetylcholinesterase) (12). The isolated material was indistinguishable from authentic aldosterone by these criteria.

RESULTS AND DISCUSSION

The results of these studies are summarized in Table 1.

Random selection of animals resulted in an equal distribution of body weights among the various experimental groups.

The mean arterial blood pressure tended in general to fall during the course of the experiments, requiring norepinephrine infusion in some instances. Three animals of the left atrial stretch group, 3 animals in the right atrial stretch group, 1 animal in the sham-operated group, and 2 of the intact animals received norepinephrine during the course of the experiment (Table 1). Examination of the steroid secretion rates in those animals receiving the sympathetic amine in the various groups failed to reveal any consistent evidence of a stimulating action of the drug on steroid secretion. This is in agreement with the findings of Newman and co-workers (13) that

TABLE 1. BODY WEIGHT, ARTERIAL BLOOD PRESSURE, VOLUME OF ADRENAL VENOUS BLOOD, BODY TEMPERATURE, AND STEROID SECRETION IN CONTROL, SHAM, AND ATRIAL STRETCH DOGS

Experimental group	Animal number	Body wt., kg.	*Average arterial b.p. during collection period, mm. Hg.*	*Volume of Adrenal venous blood, ml.*	*Average body temperature during collection period, °C*	*Hydrocortisone secretion, µg./kg. body wt./hr.*	*Aldosterone secretion, µg./100 kg. body wt./hr.*
Intact controls	1	22.0	98.7	325	37.7	42.8	50.5
	2	21.0	126.2	1060	38.8	43.0	53.9
	3*	21.0	101.6	540	39.3	60.8	42.2
	4*	21.0	113.0	560	38.5	71.5	48.9
Mean and Standard Error		21.2 ± 0.25	109.8 ± 6.2	621 ± 155	38.6 ± 0.34	54.5 ± 7.1	48.9 ± 2.4
Sham-operated Lt.	5*	15.5	85.0	200	36.4	45.2	53.7
Rt.	6	20.5	86.0	310	35.1	23.5	33.0
Rt.	7	23.5	130.0	250	40.3	21.4	41.0
Lt.	8	25.0	135.0	720	37.5	71.0	45.3
Mean and Standard Error		21.1 ± 2.1	109.0 ± 13.6	370 ± 118	37.3 ± 1.12	40.2 ± 11.7	43.3 ± 4.3
Left Atrial Stretch	9*	20.0	103.5	220	36.5	23.7	36.8
	10	22.5	107.7	100	37.3	20.6	60.5
	11*	23.0	92.7	390	35.5	18.5	50.5
	12	21.5	110.0	370	36.4	19.9	25.5
	13*	20.0	104.0	400	36.6	35.5	38.5
Mean and Standard Error		21.4 ± 0.62	103.5 ± 3.0	296 ± 59	36.4 ± 0.29‡	23.6 ± 3.1‡	42.3 ± 6.0
Right Atrial Stretch	14	26.5	91.1	—	35.8	36.7	11.9
	15*	21.5	95.0	320	37.2	18.4	26.8
	16*	21.5	125.0	350	39.3	16.8	30.7
	17*	22.0	77.1	200	37.9	6.8	29.4
	18	21.0	120.5	400	38.9	20.8	20.4
Mean and Standard Error		22.5 ± 1.0	101.7 ± 9.2	318 ± 42	37.8 ± 0.63	19.9 ± 4.8‡	23.8 ± 3.5†

* Infusion of norepinephrine in 5% glucose solution was necessary during some part of the experimental period.

† These values are significantly different from those of the sham-operated group ($p < .01$).

‡ See text for discussion of statistical validity.

norepinephrine is without effect on hydrocortisone and aldosterone secretion in the cat. The average arterial blood pressure during the period of adrenal venous blood collection was not significantly different among the various experimental groups (Table 1).

Venous pressure was measured in the external jugular vein in two additional animals by means of a saline manometer adjusted to zero at the level of the right atrium. In both animals an increase of approximately 5 cm. saline pressure was noted when the chest was opened. In one animal an additional increase of 2 cm. saline pressure was seen when the pericardium was elevated. In both animals stretching of the right atrium resulted in a decrease of 0.5 cm. saline pressure. It seems probable that the modest change in venous pressure following right atrial stretch would not have a significant effect on the experimental results, although further study is clearly needed.

The volumes of adrenal venous blood collected over the two hour bleeding periods are presented in Table 1. The average volumes obtained from the three groups of animals subjected to open chest surgery were very similar and appeared to be somewhat less than in the intact group. However, large variations were noted, and the differences between the means are not satistically significant. The higher average volume of adrenal venous blood in the intact group appears to be due to an unusually large adrenal blood flow in one animal (Table 1). Careful examination of the data failed to reveal any consistent correlation between volume of adrenal venous blood and steroid output. This is in agreement with earlier observations that the steroid secretory rate is largely independent of the adrenal blood flow within wide limits (7).

The body temperatures tended to fall during the course of the experiments. However, the average body temperatures in the open-chest sham and right atrial stretch groups, though slightly lower, are not significantly different from those of the intact controls. On the other hand, the left atrial group had a significantly lowered temperature, as compared to the intact controls. Whether this reduction (about 2.2° C) affected the results with respect to hydrocortisone secretion remains unanswered. The work of Egdahl, Nelson and Hume (14) indicates that hydrocortisone secretion is reduced in hypothermic animals, although the temperature ranges at which marked reductions in hydrocortisone secretion were observed in their experiments were much lower than are reported here. In any event, the reduction in body temperature in the left atrial stretch group was apparently without effect on the aldosterone secretion rate, since the rates of output of this steroid were within the control range.

The rates of secretion of hydrocortisone were somewhat variable. In the sham-operated group the rate of output of hydrocortisone seemed to be on the average somewhat less than in the intact control group. However, because of variation among the individual animals this apparent difference is not statistically significant. The values in the right and left atrial stretch groups are significantly lower than in the intact controls ($p < .01$) but not when compared to the more appropriate controls, that is, the sham-operated group ($P < .2 > .1$). Interpretation of the data is consequently difficult, and a final statement must await further studies using larger numbers of animals. It seems likely that if the number of animals in the sham-operated group were increased, a significant depressing effect of the open-chest procedure *per se* could be demonstrated, as well as a further depression by atrial stretch.

The rates of secretion of aldosterone are presented in Table 1. In the control animals the aldosterone secretion rate was 48.9 ± 2.45 (S.E.) μg./100 kg. body wt./hr. In the four sham-operated animals the aldosterone secretion rate was 43.3 ± 4.30. This value is not significantly different from that in the controls ($P < 0.3 > 0.2$). When the left atrium was stretched the rate of secretion of aldosterone was 42.2 ± 6.03, which is not significantly different from that in either the sham-operated or the intact control group ($P < 0.4 > 0.3$). In the dogs subjected to right atrial stretch the aldosterone secretion rate was 23.8 ± 3.46 μg./100 kg. body wt./hr. This is a highly significant reduction in secretion rate as compared to the intact ($P < .01$), sham-operated ($P < .01$), and left atrial stretch groups ($P < .05$).

Distention of the right and left atrium by external tension was chosen in favor of a balloon in the atrium as used by Henry and co-workers because it was felt that the former would permit direct visualization of the degree of distention and that it would not impede blood flow through the heart. Cardiac output determinations were not carried out in these experiments. The decrease in aldosterone secretion that was seen following stretching of the right atrium may have been mediated by a decrease in blood flow through a sensitive area on the arterial side of the vascular system, or by congestion on the venous side. If this is the case, it appears that the effects on these parameters of right atrial stretch are much different from those of left atrial stretch, since the aldosterone secretion rate was significantly different between the two groups. It is of interest that the adrenal blood flow was approximately equal in these two experimental groups, indicating that if a difference in cardiac output was actually present, it was not reflected in a change in adrenal blood flow.

The existence of nerve endings in the atria, bases of the venae cavae and pulmonary veins which could serve as stretch receptors has been documented histologically by Nonidez (15). Paintal has recorded impulses in vagal fibers which seem to respond to changes in atrial filling (16). Although it seems a reasonable possibility, it remains to be demonstrated that these receptors are concerned in the reflex suppression of steroid secretion suggested by our experiments.

Acknowledgment

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IN VITRO METABOLISM OF TRIIODOTHYRONINE[1]

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ABSTRACT

Previous studies have shown that rat kidney slices contain an enzyme which deiodinates thyroxine to yield triiodothyronine. Further, a soluble enzyme has been obtained from sonically disintegrated rat kidney mitochondria which converts thyroxine and triiodothyronine to the corresponding acetic acid analogues.

In the present study the metabolism of I^{131}-labelled triiodothyronine by intact and solubilized mitochondria of rat kidney, liver, heart and brain and by homogenates of spleen and intestine was examined.

With the intact mitochondrial enzyme system two types of reactions were observed. Deiodination of triiodothyronine occurred most actively in kidney, liver, heart, spleen and brain. Intestine showed very little deiodination. The second reaction involved the conversion of triiodothyronine to triiodothyroacetic acid. This was observed chiefly in kidney, although a small peak of triiodothyroacetic acid radioactivity was regularly observed with liver.

Sonic disruption of the mitochondria resulted in loss of the deiodinating activity and an increase in the conversion of triiodothyronine to triiodothyroacetic acid by kidney, liver, heart and brain.

The presence of the enzyme system for converting triiodothyronine to triiodothyroacetic acid in the mitochondria of organs other than the kidney increases the likelihood that it has a physiologic role in the action of the hormone. In this connection it should be noted that the tissues most susceptible to the stimulating effect of thyroid hormone (heart, kidney, liver) are the most active in this transformation.

THYROXINE is metabolized in several ways by extrathyroidal tissue. Some of these, such as the conjugation with glucuronic acid and disruption of phenoxy linkage (1) by the liver, are presumably detoxicative. The deiodination of thyroxine to triiodothyronine, on the other hand, might be prerequisite to the peripheral action of the thyroid hormone. The best evidence for this hypothesis was given by Sheehan (2), MacLagan (3) and Wilkinson (4) who demonstrated that n-butyl 4-hydroxy-3:5-diiodobenzoate which blocks the deiodination of both thyroxine and triiodothyronine, suppresses the metabolic effect of the former while enhancing the

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latter. The conversion of thyroxine to triiodothyronine has been observed repeatedly in thyroidectomized animals and in *in vitro* systems.

Further reactions involving the alanine side chain of both thyroxine and triiodothyronine have been reported. Conversion of thyroxine and triiodothyronine to their corresponding acetic acid analogues by cell free enzyme systems prepared from rat kidney mitochondria was observed (5, 6). A similar reaction has been found in the case of triiodothyronine in the intact animal (7, 8). It is not known whether such a transformation is degradative or important to the derivation of an intracellularly active hormone. Support has been given to the latter alternative by the observation that triiodothyroacetic acid is approximately 10 times more active than triiodothyronine in uncoupling oxidative phosphorylation in heart mitochondria (9), a proposed mode of action of the thyroid hormone.

If triiodothyroacetic acid is a metabolically active derivative of triiodothyronine it is probably formed *in situ* since it has been shown that following intravenous administration in the intact animal it is rapidly eliminated by the liver (10). In the original studies only kidney was used; therefore, it is important to examine other tissues for this specific enzyme system. The present paper presents experiments on the *in vitro* metabolism of triiodothyronine by various organs including brain, heart, liver, spleen, intestine and kidney.

METHODS

Materials and Preparation

Organs of female rats (Sprague-Dawley 150–200 gm.) were used in all experiments. After homogenization in sucrose the mitochondria of kidney, liver, heart and brain were isolated by the method of Schneider (11). Because of difficulty in the isolation of mitochondria from spleen and intestine, whole homogenates centrifuged for ten minutes at 4° C at 600 G were used. The soluble enzyme systems were prepared by disruption of the mitochondria of kidney, liver, heart and brain in the Raytheon 10 KC sonic oscillator for eight minutes with alcohol at −5° C as the coolant. The sonated preparation was centrifuged at 100,000 G for 30 minutes and the supernatant dialyzed against 2 liters of distilled water at 4° C for 4 hours.

Reaction Procedure

The reaction mixtures (final volume 3 ml.) contained 2.5 μ moles of DPN, 50 μ moles of phosphate buffer, pH 7.4, and 2 ml. of enzyme preparation. (Enzyme concentration: homogenate 10%; intact mitochondria 20–25 mg. of protein; solubilized mitochondria 5–10 mg. protein except for heart which was 1–3 mg. protein). To each flask was added .05 micrograms of I^{131}-labelled chromatographically pure l-triiodothyronine.[2] The reaction mixtures were incubated at 37° C for 2 hours. Enzyme preparation boiled for 10 minutes and unincubated systems were used as controls.

Separation of Products

At the end of incubation a 150 lambda aliquot of the reaction mixture was chromatographed directly. Fifty micrograms of non-radioactive triiodothyronine,[3] triiodothyroacetic acid[4] and iodide were added to the mixture as markers before application to the paper. The solvent system employed was tertiary amyl alcohol saturated with 2N NH_4OH run in descent for 24 hours at room temperature (6). The radioactivity was located in the strips by a continuously recording chromatogram scanner equipped with an end-window G.M. counter. The position of the thyronine marker compounds was determined by color development with 4-amino-antipyrine reagent (12), and iodide by a starch-iodate reagent.

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[2] Obtained from Abbott Laboratories.

RESULTS

With the intact mitochondrial enzyme system prepared from the organs studied two types of reactions were observed. As shown in Figure 1, deiodination of triiodothyronine occurred most actively in kidney and liver, with less activity in heart, spleen and brain. Intestine showed very little deiodination. The second reaction involved the conversion of triiodothyronine to triiodothyroacetic acid. As seen in Figure 1, this was observed chiefly in kidney, although a small peak of triiodothyroacetic acid radioactivity was regularly observed with liver.

With the solubilized mitochondrial enzyme systems the deiodination of triiodothyronine was sharply reduced. As shown in Figure 2, disruption of the mitochondria resulted in the loss of deiodinating activity in kidney, liver, heart, and brain. In contrast, the conversion of triiodothyronine to triiodothyroacetic acid appeared to be enhanced by sonic disintegration of the mitochondria. Comparison of Figure 1 and Figure 2 reveals the increased formation of triiodothyroacetic acid by solubilized mitochondria of kidney and liver, and the formation of a detectable quantity of triiodothyroacetic acid by the heart preparation. Brain activity remained equivocal.

DISCUSSION

Deiodination reactions involving thyroxine have been observed in a variety of tissue preparations. In rat kidney slices the major deiodination product was triiodothyronine (13, 14). Homogenates and extracts of several tissues have been studied by MacLagan (15) and Ruegamer and Yunis (16). With these preparations the deiodination proceeded more rapidly and the formation of triiodothyronine was not observed. Triiodothyronine was deiodinated apparently by the same enzyme system at a rate at least equal to the deiodination of thyroxine. Ruegamer and Yunis observed further that the deiodinating activity of their homogenate preparations was associated with the particulate matter of the cells. The present study indicates that in the case of triiodothyronine the deiodinase enzyme exists in connection with the mitochondria of kidney, liver, heart, spleen and brain. Further, this activity is dependent upon the integrity of the

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[3] Obtained from Smith, Kline and French Laboratories through the courtesy of A. E. Heming.

[4] Obtained from Hoffmann-La Roche, Inc. through the courtesy of M. J. Schiffrin.

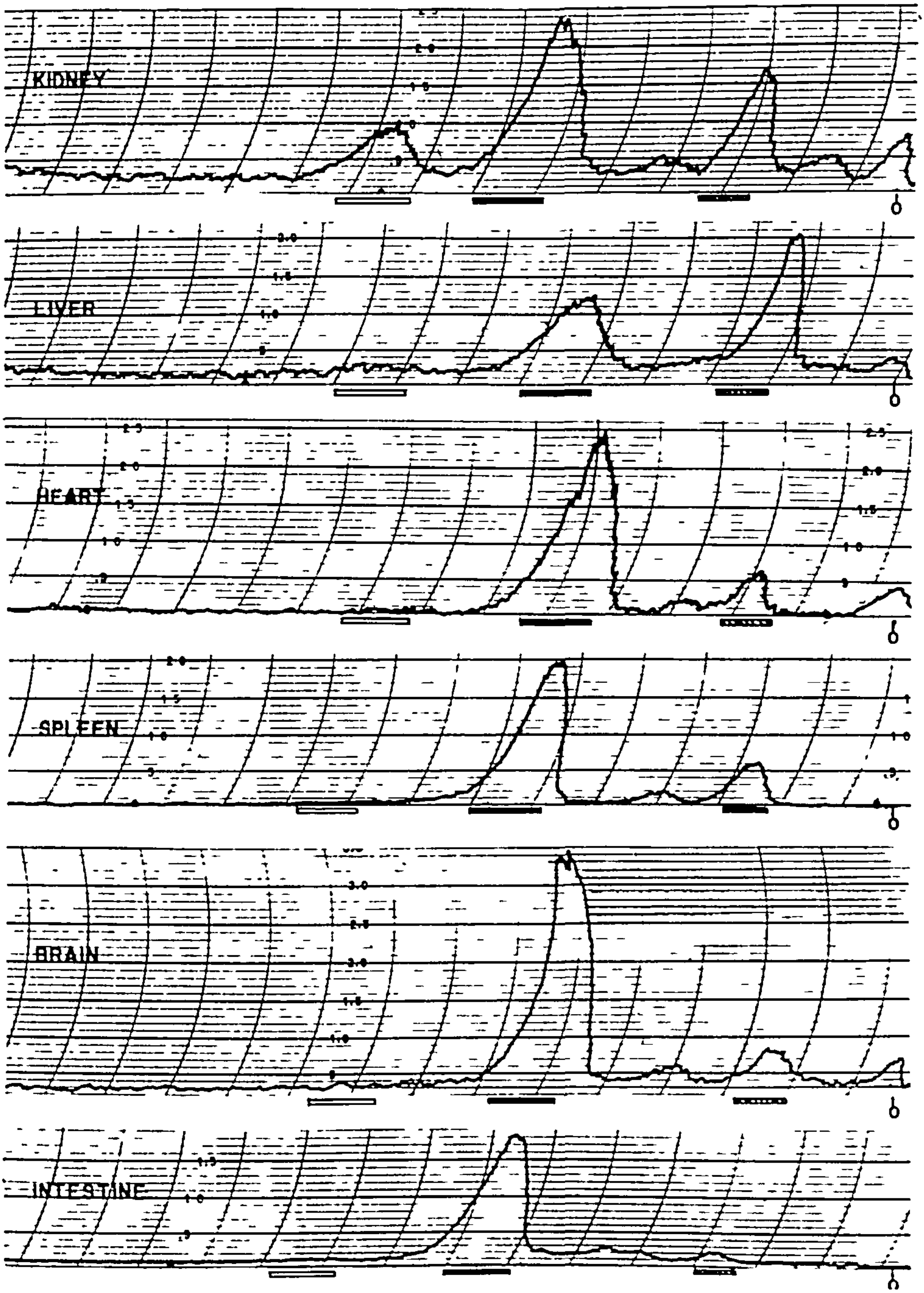

FIG. 1. Metabolism of labelled triiodothyronine by intact mitochondria of kidney, liver, heart and brain, and by homogenate of spleen and intestine. Position of non-radioactive marker iodide shown by cross hatched bar, triiodothyronine by solid bar, and triiodothyroacetic acid by open bar. O—origin of chromatogram. Chromatographic front not shown.

mitochondria as deiodination is sharply reduced following sonic disruption of these particles. The loss of deiodinating activity attendant upon disruption of the mitochondria is believed not to be due to dilution effect, as was reported by Tata (17) working with dog brain suspension, since the same weight of tissue was used in preparation of both intact and solubilized mitochondria. Failure to demonstrate deiodination in previous experiments with intact mitochondria was probably due to the extraction of the reaction mixture with butanol prior to chromatography.

If the deiodinating enzyme observed in the slice experiments and in the cell free preparations is the same, the accumulation of triiodothyronine in the intact cell suggests the presence of a trapping system which is de-

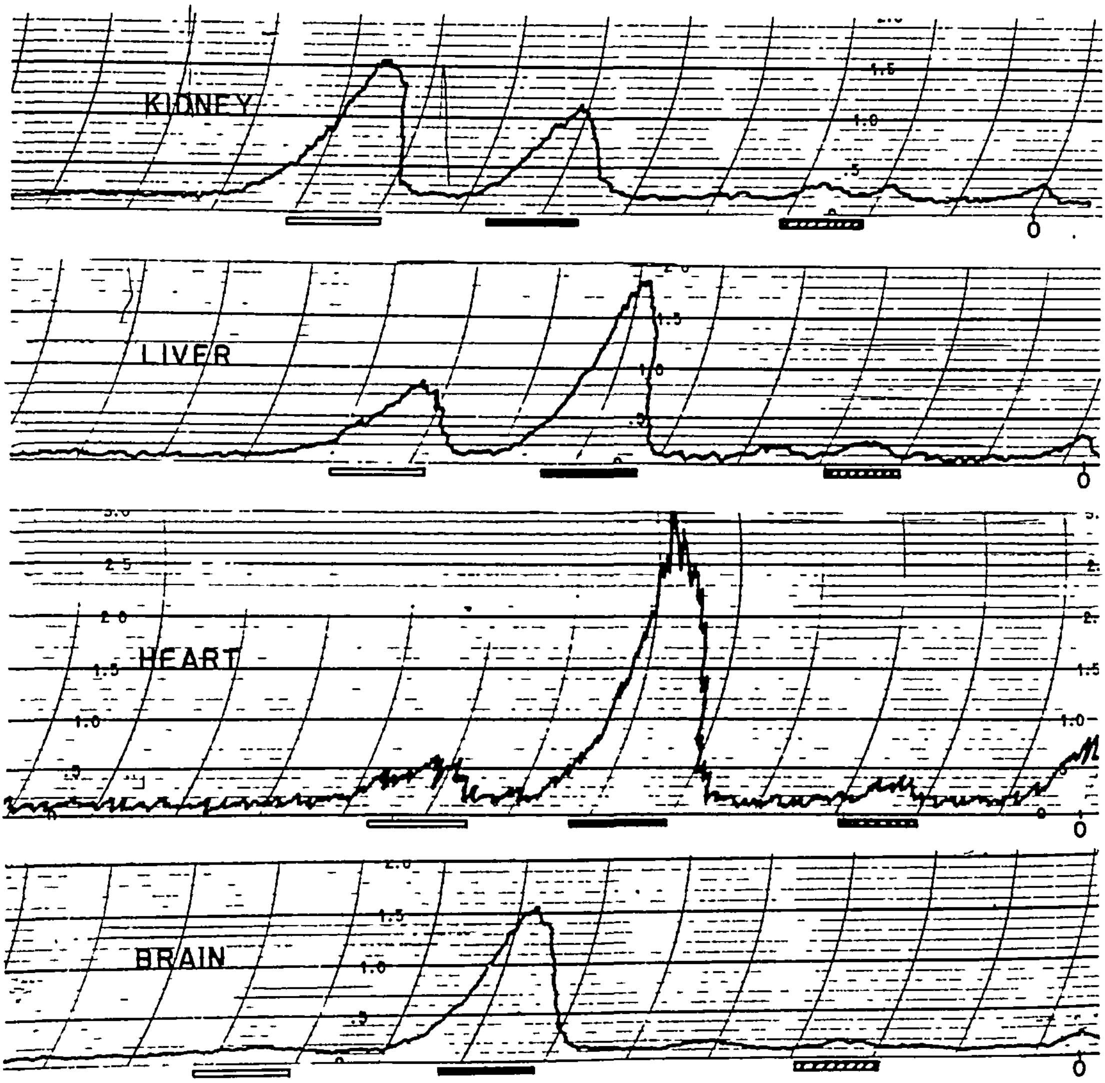

Fig. 2. Metabolism of labelled triiodothyronine by solubilized mitochondrial enzyme system of kidney, liver, heart and brain. Position of non-radioactive marker iodide shown by cross hatched bar, triiodothyronine by solid bar, and triiodothyroacetic acid by open bar. O—origin of chromatogram. Chromatographic front not shown.

stroyed by the disruption of the cell. Within this hypothesis, such a system might provide a self regulatory mechanism within the cell. The accumulation of triiodothyronine would enhance the metabolic activity of the hormone, either *per se* or as a precursor of an active compound derived possibly through alterations in the alanine side chain. Deiodination of triiodothyronine, on the other hand, would result in loss of hormonal activity.

The enzymatic formation of triiodothyroacetic acid from triiodothyronine was first observed using rat kidney mitochondria (5). A preliminary survey of other organs of the rat failed to reveal similar activity. The use of intact mitochondria or homogenates in the initial survey probably accounted for the failure to observe triiodothyroacetic acid formation. It is clear from the present study that disruption of the mitochondria enhances the triiodothyronine to triiodothyroacetic acid conversion. This may be accounted for at least in part by the destruction of the deiodinating system by sonic disruption, which in intact mitochondria competes for the labelled substrate. In liver, where deiodinating activity was the greatest, for example, it was difficult to demonstrate triiodothyroacetic acid formation with intact mitochondria, whereas with the solubilized enzyme rather large amounts of triiodothyroacetic acid were produced.

The presence of this enzyme system in the mitochondria of tissues other than kidney, together with the observation that intravascular triiodothyroacetic acid is rapidly eliminated (10), makes it appear unlikely that triiodothyroacetic acid is formed in the kidney for use elsewhere in the body. It appears more likely that many tissues can carry out this conversion. The presence of this enzyme system in these tissues increases the likelihood that it has a role in the metabolism of the hormone. That this role may be physiologic is suggested by the observation that tissues most susceptible to the stimulating effect of thyroid hormone (heart, kidney and liver) (18), are most active in converting triiodothyronine to triiodothyroacetic acid. It must be emphasized that the presence of triiodothyroacetic acid in this metabolic sequence does not necessarily mean that it is the active hormone, since it may well be a precursor or product or one of many hormones.

Acknowledgment

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PATHOLOGIC CHANGES IN ADRENALECTOMIZED, SENSITIZED RATS TREATED WITH ADRENAL CORTEX EXTRACT[1]

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ABSTRACT

Male rats of the Sprague-Dawley strain were adrenalectomized and unilaterally nephrectomized at an initial weight of about 300 gms. The rats were adapted to the tube-feeding of a medium carbohydrate diet which contained 4% added NaCl by dry weight. The following doses of adrenal cortex extract (ACE) were given by subcutaneous injection for 56 days: 0.0, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 8.0 cc. per rat per day. When the dose of ACE was 1.0 cc. per day or greater the blood pressure was elevated in proportion to the dose. Doses of 4 and 8 cc. of ACE per rat per day caused an increase in the amount of renal and myocardial pathology. Arteriosclerosis was not seen in any animal of the present study but has been seen in a small number of sensitized rats given large doses of ACE in other experiments.

A NUMBER of sodium-retaining synthetic steroids cause damage to the kidney and to the cardio-vascular system when administered to experimental animals in high dosage. Damage is more likely to result when the animal is sensitizied by the removal of one kidney and by the administration of a high load of sodium chloride (1). Ingle and Baker (2) observed renal and cardiovascular damage in sensitized rats given large doses of adrenal cortex extract (ACE) and in subsequent experiments (3, 4) we have observed some elevation in blood pressure and some pathologic changes when doses of ACE nearer the physiological range were given to the sensitized rat.

This is a report on the dose-response effect of ACE upon blood pressure and tissue changes in the adrenalectomized-sensitized rat. The data show a significant relationship between the dose of ACE and the extent of the changes from normal.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of approximately 300 grams. They were then adapted to the tube-feeding of a fluid high sodium chloride diet (Table 1) in the amount of 13 cc. each morning and late afternoon. Following adaptation to the diet all of the rats were subjected to unilateral nephrectomy plus bilateral adrenalectomy. Clean but not sterile

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technique was used. Immediately following operation each rat was given 5000 units of penicillin and 5 mgm. of streptomycin. This was not continued. Some of the rats were kept without replacement therapy; the others were given varying doses of beef adrenal cortex extract (Upjohn), 1 unit per ml., in aqueous solution (ethanol-free) by subcutaneous injection twice daily for a period of 56 days.

For the determination of blood pressure the rats were anesthetized with ether and the abdominal aorta was cannulated below the level of the renal arteries. The cannula was attached by a short length of fine-bore rubber tubing filled with heparinized saline to a mercury manometer. The record was taken when anesthesia was light, as judged by retraction of the rat's hind foot on pinching.

All rats were killed by exsanguination under ether anesthesia at the end of 8 weeks. Autopsy was performed and gross changes noted. The heart, kidney and portions of liver, spleen, pancreas, lung, stomach and intestine were fixed in 10% neutral formalin.

TABLE 1. HIGH SALT, MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour	60
Salt mixture U.S.P. XIV	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil plus 100 mg. K	10
Mazola oil	190
Casein	160
Starch	300
Dextrin	150
Sucrose	140
Sodium chloride	50

Hematoxylin and eosin was the routine stain and selected examples of heart and kidney were examined by the periodic acid-Schiff routine, and Weigert's resorcin-fuchsin stain for elastic tissue.

EXPERIMENTS AND RESULTS

ACE was administered in amounts ranging from 0.1 cc. per rat per day to 8 cc. per rat per day. Dosage in relation to results is shown in Table 2. Eleven adrenalectomized-sensitized rats were kept without ACE; the high salt diet supported life.

Survival. One rat died with symptoms of adrenal cortical insufficiency. Five rats died after becoming completely anuric. The body cavities were filled with fluid. The ureter of each of the 5 rats was found to be obstructed by gravel, the kidney of each had developed some degree of hydronephrosis and the pelvis contained gravel. We have no insight into the reasons for formation of the kidney stones but noted that death occurred only in the rats which were either treated with small doses of ACE or were untreated.

Growth. The higher doses of ACE caused some suppression of the amount of gain in weight and there was some suppression of hair-growth. The animals treated with moderate doses of ACE gained more weight than the untreated animals. There was some atrophy of thymus when the dose of ACE was large and an increase in the size of thymus when the dose of ACE was small or when none was given.

Blood pressure. The blood pressure was elevated above the average for untreated rats when the dose of ACE was 1 cc. or greater per rat per day: the extent of increase was related to the size of the dose. The average values for all of the sensitized rats of this study was higher than the average value for normal rats.

TABLE 2 EFFECT OF ACE IN ADRENALECTOMIZED-SENSITIZED (UNILATERAL NEPHRECTOMY, 4% NaCl DIET) FOR 56 DAYS TUBE-FEED 13 CC 2×DAY
AVERAGES AND STANDARD ERRORS

Experimental conditions	No rats	Gms body weight		Organ wts, mgm			Mm Hg BP	Ratings of pathology*		
								Kidney		Heart
		Initial	Final	Heart	Kidney	Thymus		Gross	Micro	Micro
8 cc ACE day	6	311 ±4.0	381 ±4.6	1370 ±28.3	2358 ±91.2	220 ±16.9	181 ±7.5	2.7 ±0.4	2.67 ±0.41	1.0 ±0.22
4 cc ACE day	6	313 ±2.6	393 ±3.9	1380 ±28.6	2245 ±82.4	290 ±18.5	159 ±5.0	2.3 ±0.4	2.50 ±0.37	0.67 ±0.20
2 cc ACE day	6	319 ±3.6	415 ±8.8	1313 ±31.1	2025 ±90.1	549 ±79.8	146 ±5.9	0.7 ±0.02	1.08 ±0.26	1.30 ±0.25
1 cc ACE day	7	313 ±4.4	397 ±5.3	1197 ±40.5	1839 ±43.0	543 ±48.7	139 ±1.0	0.6 ±0.01	2.00 ±0.51	0.28
0.5 ACE day	4	313 ±1.3	418 ±4.2	1245 ±30.7	2077 ±46.4	634 ±87.3	128 ±4.5	0.12 ±0.01	1.25 ±0.20	0.00
0.2 ACE day	4	321 ±4.2	430 ±4.9	1267 ±18.4	2002 ±58.1	739 ±60.5	130 ±3.2	0.38 ±0.02	0.50 ±0.19	0.00
0.1 ACE day	6	319 ±3.5	412 ±5.0	1195 ±36.6	1885 ±90.1	676 ±45.1	129 ±1.4	0.58 ±0.08	0.50 ±0.21	0.33
No ACE	11	316 ±2.9	402 ±4.9	1154 ±16.9	1767 ±40.8	658 ±40.8	131 ±1.7	0.73 ±0.17	0.91 ±0.27	0.18
Normals low salt diet	6	308 ±2.5	408 ±3.8	1130 ±20.2	1335 ±25.4	462 ±34.1	122 ±1.3	0.0		

* The ratings of gross renal pathology are based on the following scale 0 = no abnormality 1 = few tiny nodules, 2 = moderately nodular with some mottling, 3 = more nodules mottling with some gray spots 4 = rough mottled with gray and red spots, and 5 = changes listed previously more severe giving flea bitten grayish-yellow appearance

The ratings of the micro ... NEY 0 = no abnormality
1 = hypertrophy of glomeruli ... mmation 2 = active pelvic
inflammation, 3 = glomerular ... ruli sclerosis of arterioles
and 5 = necrosis of glomeruli ... f muscle 2 = focal inflam
mation or fibrosis of myocardi ... = necrosis of myocardium
and or vessel walls

Heart. The average weight of the heart was greater in the rats given large doses of ACE. One rat given 8 cc. of ACE daily had visible gray spots in the wall of the heart.

As in previous experiments (3, 4, 5), the microscopic changes in the heart were assessed on the basis of muscle hypertrophy (grade 1), the presence of granulomatous or fibrotic foci in the myocardium (grade 2), and hypertensive damage to the coronary blood vessels (grades 3 and 4; footnote to Table 2). Changes in the heart were more common in the rats treated with 8, 4 and 2 cc. of ACE (Table 2). The cardiac muscle cells hypertrophied and in some rats this was associated with focal lesions of the myocardium. They consisted of collections of macrophage cells, Anitschkow myocytes, and fibroblasts within the interstitial tissue (Fig. 1). Other rats showed focal myocardial fibrosis, sometimes in relation to branches of the coronary arteries. However, despite the record of hypertension obtained with the higher levels of ACE administration there was no evidence of hypertensive vascular damage to the arteries of the heart in any rat.

Kidney. There was a positive relationship between the dose of ACE and the average weight of the kidney. At doses of 4 and 8 cc. of ACE per rat per day there was an increase in the amount of gross damage consisting in mottling, roughness due to pits and nodules, and some kidneys had gray spots and red spots. At doses of 2 cc. and less the damage was minimal.

As in previous experiments (3, 4, 5) the degree of microscopic damage to the kidney (footnote, Table 2) was assessed on the basis of focal inflammation in the cortex or pelvis (grades 1, 2), glomerular tuft lesions (grade 3), and the association of glomerular damage with hypertensive changes in the renal arterioles (grades 4, 5). Small focal areas within the cortex showing periglomerular fibrosis, a few casts with atrophy of tubular epithelium, and some round cell infiltration were present in some rats in all the experimental groups. Active chronic inflammation of the renal pelvis was seen in 9% of the rats, but this was not confined to animals of any one group. Glomerular tuft lesions occurred in rats treated with 8, 4 or 2 cc. of ACE (Table 2), but examples of this type of damage were seen in 2 rats maintained on the salt diet without hormone. The glomerular lesions consisted of an increase in intercapillary hyaline material. In some instances the tuft was shrunken and the subcapsular space enlarged, while in others the hyalinized tuft adhered to a thickened Bowman's capsule so obliterating the space (Figs. 2 & 3). Although the blood pressure in rats treated with higher doses of ACE was elevated there was no evidence in

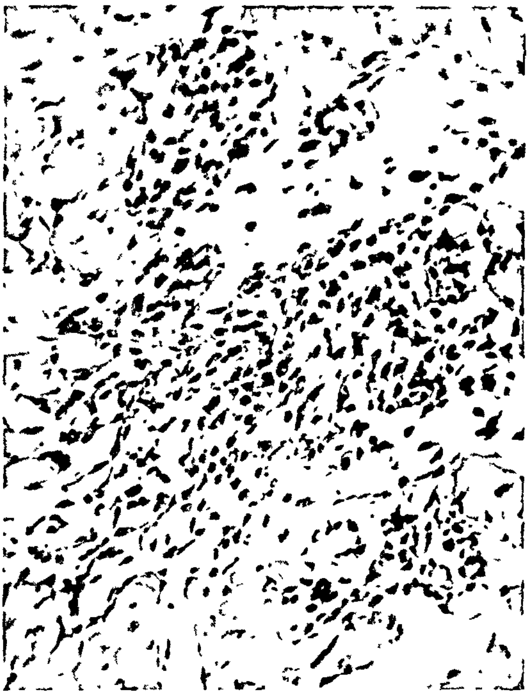

FIG. 1. Heart. ACE 8 cc. daily. The cardiac muscle fibres are separated by a focus of fibroblasts and macrophage type cells. B.P. 195 mm. Hg. Haematoxylin and eosin ×225.

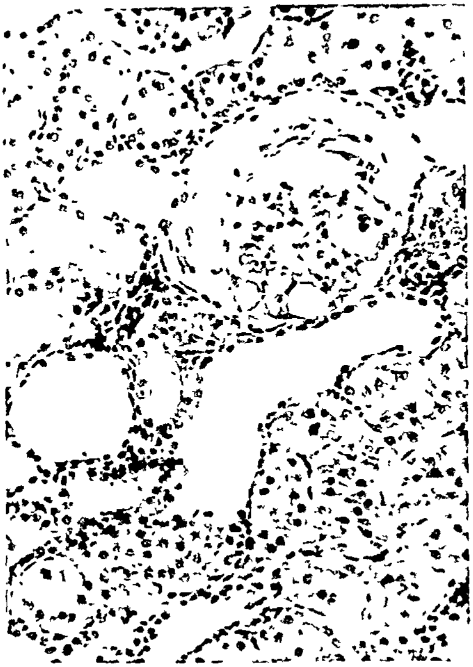

Fig. 2. Kidney. ACE 4 cc. daily. The glomerulus is partly hyalinized and adherent to Bowman's capsule. The tubules are dilated. B.P. 158 mm. Hg. H.&E. ×185.

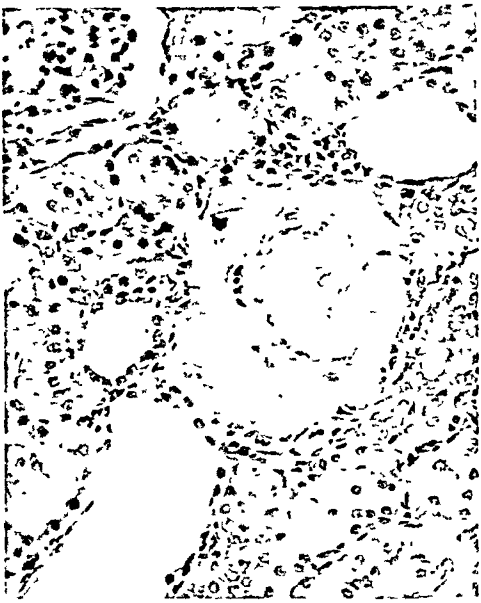

Fig. 3. Kidney. ACE 4 cc. daily. The glomerulus is almost completely sclerosed and the subcapsular space is obliterated. The tubules are dilated. B.P. 150 mm. Hg. H.&E. ×250.

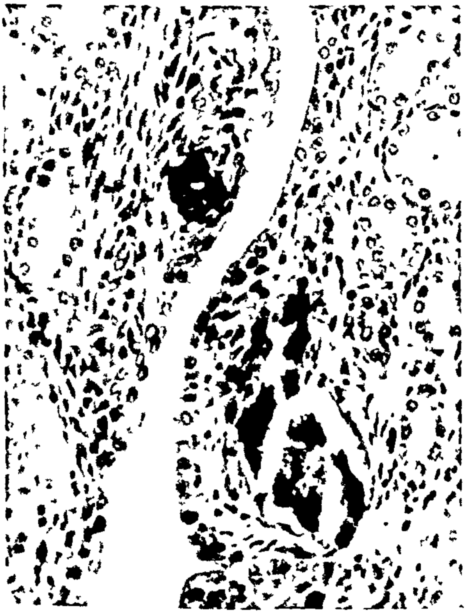

Fig. 4. Kidney. ACE 4 cc. daily. Deposits of calcified debris are present beneath the epithelium of the renal pelvis. H.&E. ×270.

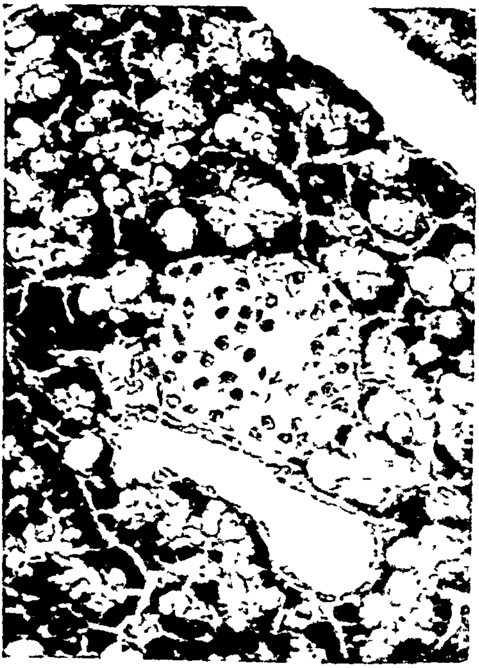

Fig. 5. Pancreas. ACE 8 cc. daily. "Signet-ring" cell change is present in the acinar tissue. H.&E. ×295.

these experiments of hypertensive arteriolosclerosis or arteriolonecrosis affecting the arterioles of the kidney. In view of the deaths at the beginning of these experiments due to renal stone and hydronephrosis, it is of interest to record that 9 of the rats killed at the end of the investigation showed deposits of calcium salts beneath the epithelium of the renal pelvis (Fig. 4). Examples were seen in animals treated with ACE at several dose levels, including the higher range, and also in one rat maintained only on the diet.

Other organs. Hypertensive vascular lesions were absent in the other organs taken for examination. Eight rats showed a focal "signet-ring cell" change in the pancreatic acinar tissue (Fig. 5). The significance of this finding is not clear, but it has been described by Salgado and Selye (6) in the rat following treatment with methylandrostenediol. In our experiments it occurred in rats treated with ACE and in 1 rat maintained solely on the salt diet.

DISCUSSION

We anticipated some similarity in the kinds of tissue damage in this and in related studies (3, 4, 5). Unlike our previous results we failed to reproduce hypertensive arteriosclerosis, although hypertensive records were obtained manometrically in the present experiment. The rise in blood pressure and the extent of pathology showed a positive relationship to the dose of adrenal cortical extract. Untreated sensitized rats showed an average blood pressure higher than normal average and there was more microscopic damage to hearts and kidneys than occurs in normal animals on a normal intake of sodium.

It may be appropriate here to discuss more fully the relationship of the pathological findings in the kidney to the rise in blood pressure. The most common finding in animals of all groups was foci of dilated tubules containing protein casts and lined by a rather flattened epithelium with basophilic cytoplasm. In many cases the related glomerulus showed periglomerular fibrosis and an increase of lymphocytes was present in the adjacent interstitial tissue (grade 1 lesion). Such changes have been described in the kidney of the aged rat by Ingle and Baker (2) and more recently by Kennedy (7). The latter has shown that they develop at a rather earlier age, between one year and 18 months, in the unilaterally nephrectomized rat. Using much younger rats we have considered that these appearances are a result of focal chronic pyelonephritis although the mechanism of their production may be more intricate. They are not necessarily associated with obvious ascending infection in the medullary tubules.

Glomerulosclerosis is also seen in the aging rat. In the present experiments it occurred most frequently in those rats treated with the higher doses of adrenal cortex extract in which hypertensive records were obtained, but hypertensive arteriosclerosis was not seen in any animal. We have no firm conviction that glomerulosclerosis alone (grade 3 lesion) can be regarded as hypertensive in nature in the rat. Hypertensive vascular damage has been seen in a few individual sensitized rats given ACE in earlier experiments (3, 4). Here the rats were allowed to eat the 4% sodium chloride diet *ad libitum*, but the daily intake of the group was measured. The actual dietary intake of individual rats was not controlled.

Small doses of ACE, as little as 1 cc. per rat per day, cause an elevation of blood pressure in the sensitized-adrenalectomized rat. Rats given 1 and 2 cc. of ACE per rat per day have thymus glands which are heavier than average, a sign of insufficiency of thymolytic steroid in this dosage of ACE. Elsewhere (3, 4) we have noted that the non-adrenalectomized rat given a high salt load may suppress its secretion of sodium-retaining steroids without a concomitant decrease in secretion of glucocorticoids. The adrenalectomized rat given a constant intake of ACE is at a disadvantage in dealing with a high load of salt; an excess of sodium-retaining steroid may coexist with a relative deficiency of glucocorticoids.

It is tempting to compare the effects of ACE in the sensitized rat with the effects of adrenal enucleation in the sensitized rat, but it is not shown that the administration of ACE can fully simulate the effects of adrenal enucleation which include severe hypertension and arteriosclerosis. The regenerating enucleated adrenal has not been found to secrete a greater than normal amount of sodium-retaining steroid (8). It is conceivable that the regenerating adrenal causes trouble by failing to suppress its secretion of sodium-retaining steroid to a minimum as can the normal adrenal which adjusts to a high salt load. Studies of the pattern and amounts of steroid secreted by the regenerating adrenal should include a time-response comparison of steroid output by normal and regenerating adrenals at both high and low salt loads.

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PHASE RELATIONS OF 24-HOUR PERIODICITIES IN BLOOD CORTICOSTERONE, MITOSES IN CORTICAL ADRENAL PARENCHYMA, AND TOTAL BODY ACTIVITY[1,2]

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ABSTRACT

Under conditions standardized for analysis of 24-hour periodicity, with light from 06:00 to 18:00 alternating with 12 hours of darkness, corticosterone in mouse serum undergoes reproducible 24-hour changes, with peak blood levels at about 16:00. Significant differences in phase characterize the 24-hour rhythms in serum corticosterone and in mitoses of cortical adrenal parenchyma; under the same lighting conditions, the peak of the latter occurs at about midnight. The corticosterone rhythm leads in phase the rhythm in gross motor activity and lags behind the eosinophil rhythm, in the absence of intentional stimulation other than daily routine. As compared to the significant change in blood corticosterone level along a 24-hour scale, the immediate effect of audiogenic stimulation upon serum corticosterone is slight. It consists of a small rise following stimulation at the time of the daily "corticosterone low" and is negligible after stimulation at the time of "corticosterone high." The daily rhythm in blood level of adrenal 11-oxycorticoid in nocturnal mice, as compared to predominantly diurnal man is comparable in amplitude but differs in its environmental phase relations, indicating a species difference in mode of synchronization of rhythm.

DAILY changes in corticosterone content of mouse serum were studied by adapting several chemical methods (1–3) for use on small samples. The occurrence and the timing of daily changes in blood corticoid of mice had earlier been suggested from eosinophil behavior under various standardized conditions (4–9) and blood-corticoid periodicity had been interpreted as underlying a 24-hour adrenal cycle, traced to the gland itself by

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spot-checks of mitotic counts in its parenchyma (10, 11). Herein, the periodicity and phase of adrenal mitoses in relation to rhythms in blood corticosterone and eosinophils will be described on the basis of more frequent sampling along a 24-hour scale, as a possible time base for future studies of hormone production and release. Since corticoids in the urine or blood of man or monkey exhibit 24-hour variations (12–15), corresponding changes in nocturnal rodents are also of interest to comparative endocrinology. In man, morning eosinopenia (16), as well as a rise in blood corticoids (17), is initiated before the habitual time of awakening. The phase relations of the rhythms in blood corticosterone, on one hand and in gross body activity on the other, in mice studied under standardized circumstances thus deserve interest. Will the daily rise in blood corticosterone of mice lead the onset of gross body activity, as an endocrine entity may do (7, 8), or will it lag behind as a mere "reaction to activity?" The suggestion that an adrenal cycle is preparatory for daily activity and that its period does not depend directly or solely upon environmental control (5–8, 18) differs from unqualified references 1) to daily changes in corticoid levels as "responses to the stresses of daily life" and 2) to a "basal" level of adrenal cortical activity as a characteristic of rest and/or sleep.

The role of the adrenal cycle, in functional integration and environmental adaptation has been discussed (7, 8, 18); this endocrine entity persists under conditions which obliterate the estrus cycle of mice (5) and it develops earlier than the menstrual cycle in human beings (19). The main point of this paper is a description of the more detailed phase relations of some parameters of the adrenal cycle in mice, a documentation of mechanisms remaining beyond our scope.

MATERIALS AND METHODS

Inbred mice of the C(Bagg albino) and the D_S (Dilute Brown, Subline S) stocks were used, sex and age being given with the results. Blood eosinophil counts were done with assembly-line procedures (21), on separate groups of mice, killed at 4-hour intervals during a 24-hour period. From the same mice, after cervical dislocation, the adrenals were removed and mitoses in cortical parenchyma were counted in histologic sections, 5 μ thick, stained with Heidenhain's iron hematoxylin and faintly counterstained with eosin. The mitotic counts were done under oil immersion, in nearly equatorial sections of the cortex (at least 10 μ apart from each other). Blood corticosterone was determined on ∽0.5 cc. of serum, pooled from groups of 5–15 mice. Two methods were used, a double isotope derivative assay (2), briefly P, and a fluorometric technique (3), briefly S; method S is known to lack the specificity of P, which is more rigorous but more time consuming than S. The much higher absolute values for corticosterone obtained (even on true duplicates) with S cannot be interpreted as such and will be discussed only after conversion into "relatives" (% of 24-hour mean) (cf. Fig. 1).

Corticosterone would appear to be the major steroid product secreted by the mouse adrenal cortex.[4] Bush (22) had earlier reported that in the rodent, the principal adreno-

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[4] For a detailed analysis of steroids in mice bearing an ACTH producing tumor see reference 31.

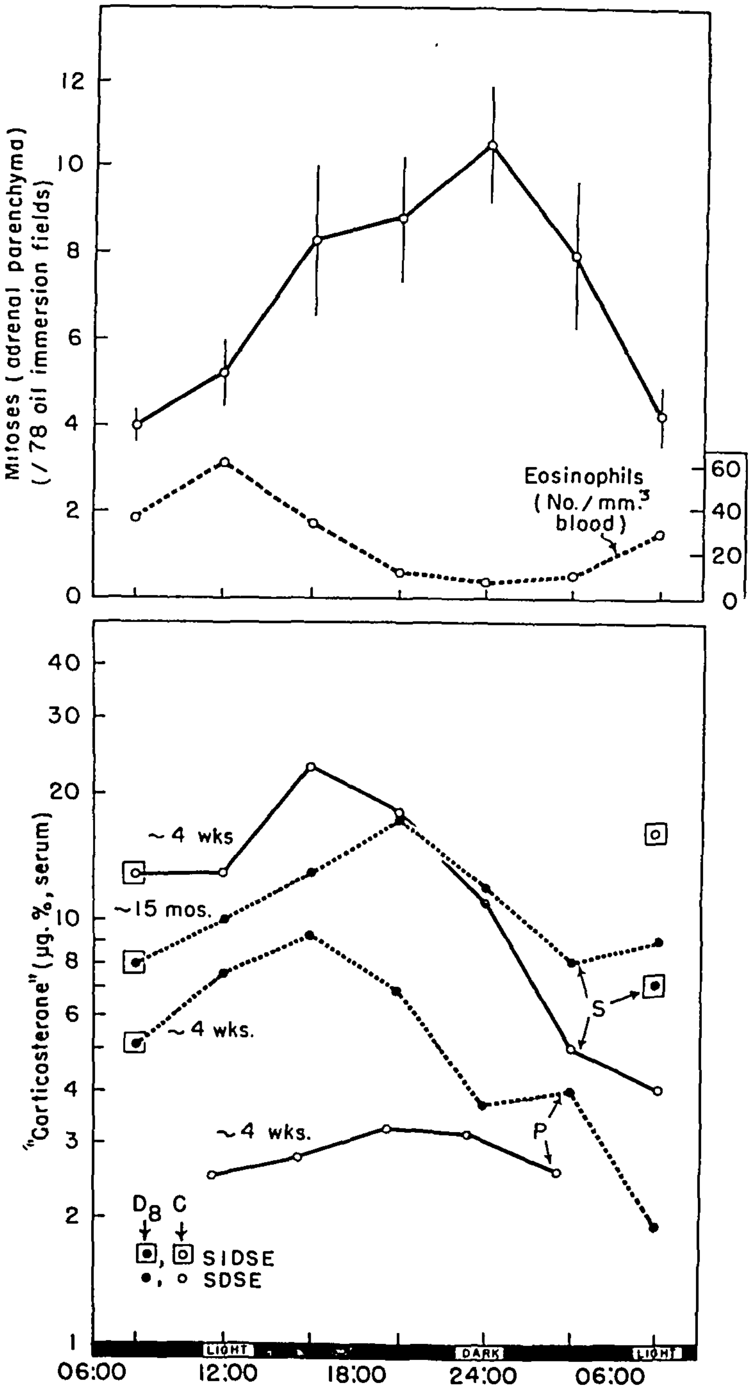


Fig. 1. Some parameters of the daily adrenal cycle in the mouse. Absolute values. Top: Mitoses of adrenal cortex and blood eosinophils, in male C mice, 4 weeks of age. Bottom: 24-hour changes of corticosterone, determined by different methods, P and S (see text and Figure 2). Each point based upon separate groups of mice (serially independent sampling as to animals). SIDSE = serially independent sampling as to environment (groups of mice from experimental rooms not entered during the 24 hours prior to sampling); SDSE = serially dependent sampling as to environment (for a discussion of such sampling procedures see reference 18).

cortical steroid is corticosterone; he could not detect any hydrocortisone. One of the present authors (Ralph E. Peterson) has assayed pooled mouse serum by the isotope derivative and an isotope derivative dilution method and found 7 μg.% corticosterone, but no hydrocortisone. In addition, isotope derivative and isotope derivative dilution assays have been performed on mouse adrenal vein blood. (Dr. William Tullner kindly carried out the adrenal vein cannulations in the mice.) Hydrocortisone could not be detected. Corticosterone levels ranged from 500 to 700 μg.%, and aldosterone levels were 10 to 20 μg.%.

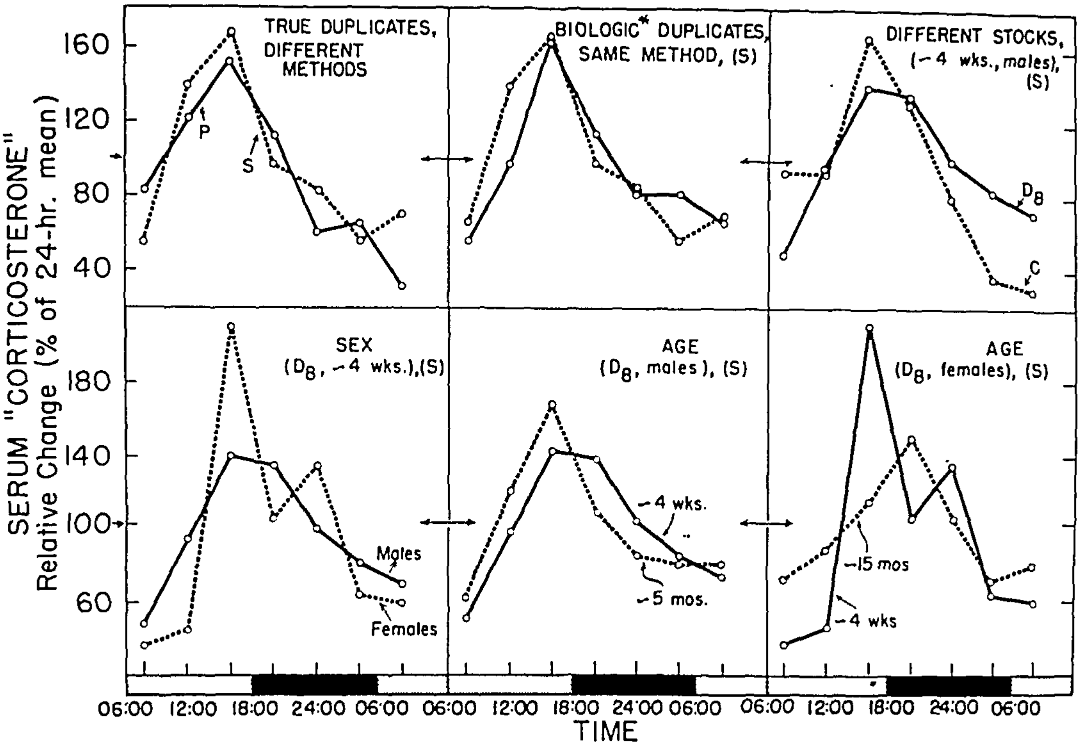
Body activity was recorded in ten electronic actometers, consisting of cages mounted on vertically compliant suspensions, resonant at 90 hertz, which actuated motors from electro-dynamic speakers to provide velocity-sensitive transducers (23). Electrical outputs were amplified and after amplitude discrimination actuated digital registers, which were simultaneously photographed at hourly intervals.

All of the experiments reported herein were carried out on singly-housed animals with Purina dog chow and tap water available *ad libitum* from weaning and throughout the investigation. For nine days prior to each study, and throughout the investigations, the mice were kept at 24.5 ± .5° C, in light from 06:00 to 18:00, alternating with twelve hours of darkness.

RESULTS AND DISCUSSION

Figure 1 shows the absolute values obtained for mitoses in cortical adrenal parenchyma, for blood eosinophils and for serum corticosterone. On the curves for corticosterone, each point represents one determination on a serum pool from a group of animals, killed at a given time of day. By contrast, the curves of eosinophils, mitoses, and body activity consist of points representing a mean value, computed from the individual data. As group phenomena, the 24-hour rhythms studied stand out clearly in Figure 1; only the curve for corticosterone at the bottom of this figure seems flat, but this curve is incomplete, since the samples at 08:00 were inadvertently lost; the lack of data at the usual time of minimum value may have contributed to the lesser amplitude of that curve.

These studies were carried out under standardized conditions, but earlier reference to the absence of intentional stimulation other than daily routine deserves comment. It is true that separate subgroups of comparable animals were studied at different time points. But mice used later in the day were unintentionally disturbed by 1) noise which could not be avoided while removing the mice studied earlier from their cages, and by 2) the disruption of the dark period by the use of a shaded flashlight (for locating the animals). Moreover, visual and other stimulation (e.g., that associated with the presence of the experimentor in the mouse room) also can affect the experimental animal (24, 25). Thus, by using different mice at different time points, only serial independence as to animals was achieved while the effect of serial dependence as to experimental room remains to complicate the data. As a spot-check on the later effect (serial dependence as to environment), in some of the series on corticosterone, at the last time point (08:00), comparable animals housed in a different but comparable room also were studied and such data are included in Figure 1. At 08:00, the end of study, corticosterone values were usually, but not invariably, lower in mice disturbed by prior sampling at 4-hour intervals than in animals kept undisturbed in a different room. The effect of unintentional stimulation of mice by the experimentors was not analyzed statistically in the limited number of pertinent corticosterone pools, but it was highly significant in comparable data on several other rhythms (e.g., mitoses in ear epidermis).



*Pools from comparable groups of mice sacrificed at comparable times during the same 24-hr period.

FIG. 2. Daily change in serum corticosterone of mice. "Relative" values (% of 24-hour mean).

Figure 2 includes additional data on corticosterone in mouse serum, all expressed as per cent of 24-hour mean. Reasonable agreement between the daily changes depicted by methods P and S may first be seen from determinations on true duplicate samples. Even though, in absolute terms, higher values are obtained by S than by P, almost certainly due to the lesser specificity of S, the curves yielded by the two methods become comparable after conversion of data to per cent of series mean. "Biologic" duplicates, i.e., two sets of determinations made by method S on two subgroups of comparable mice, killed at 4-hour intervals during the same 24-hour period are also in good agreement. Figure 2 shows, further, the relative curves of "corticosterone" for animals of different sex, but of the same stock and age, as well as stock and age comparisons (some curves are reproduced in several comparisons). If, as seems likely, "relative" results obtained by S depict changes in corticosterone level, it can be suggested first, that in mice of two stocks, of both sexes and of widely different ages, 24-hour changes characterize the level of corticosterone in blood; and second, that the daily peak in serum corticosterone of mice occurs at about 16:00, under conditions of light from 06:00 to 18:00 alternating with twelve hours of darkness (for the data on D_8 females, ~15 months of age, see discussion below).[5]

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[5] The two groups of mice were different in age, sex, stock, and in that the D_8 mice had the Bittner mammary tumor agent, while the C mice did not have the agent.

As compared to the 24-hour periodic changes in serum corticosterone, occurring in mice on a standardized daily routine, the extent of change following exposure to audiogenic stimulation was small. Noise-sensitive D_8 mice, about five weeks of age, were studied in a specially constructed stimulator;[6] at two phases of daily cycle, they were exposed for 60 seconds to noise of about 117 db (above 0.0002 dynes/sq.cm. r.m.s. pressure), yielded by a noise generator. Individual animals were transferred from their cages to the stimulator within less than 30 seconds. Upon completion of stimulation, the animals were killed and pools of serum collected for corticosterone determination. Serum from animals with and without audiogenic convulsions was pooled separately, as was serum from comparable animals not subjected to audiogenic stimulation. Determinations were done with P and S, on separate sets consisting of the three types of pools compared; the results of the two methods expressed as % change from the nonstimulated value were roughly in agreement.

Stimulation in the morning, not followed by convulsion, had a slight effect: an average increase in serum corticosterone of 19.6 ± 6.9 per cent was found for three pools obtained after stimulation, as compared to three paired pools from nonstimulated mice, when the latter values were equated to 100 per cent. By contrast, five similar comparisons of values after stimulation in the evening, not followed by convulsion, with corresponding values on nonstimulated mice revealed no effect: two pools after stimulation were higher, two were lower, and one was equal to a paired pool from nonstimulated animals. With the value for nonstimulated mice equated to 100 per cent, the corresponding difference of −2.8 ± 16 seems negligible. Furthermore, following stimulation in the evening, three pools also were collected from animals exhibiting audiogenic seizures. The mean difference between the corticosterone level in these pools, on one hand, and that in paired pools from nonstimulated animals was negligible, i.e., −4.8 ± 13 per cent.

Figure 3 shows 24-hour changes in gross body activity of two different populations of mice kept in different but comparable rooms; the sensitivity of the actometers was adjusted to record "washing" movements as well as locomotion. As was anticipated (26–28), both curves exhibit significant periodicity, but the peaks occur at different times. Each curve is based upon data from five mice, in individual actometers, kept in a room housing also comparable animals used for the collection of serum for corticoid determinations. Each activity value represents an integration of actometer counts over a given 4-hour period and is plotted at the midpoint of that period. The corticosterone values describe discrete 4-hourly samples; peaks or troughs lying between the times chosen for sampling thus may have been obscured. This limitation notwithstanding, it is of interest to counterpose to the curve in body activity, the corresponding curve for blood "corticosterone" (S). Such a comparison in Figure 3 suggests that a) the curve for "corticosterone" rises before that for body activity, b) the peak for "corticosterone" precedes that for body activity, and that c) "corticosterone" levels have dropped below their day mean at a time when body activity is still above this mean (100 per cent line, Fig. 3). Along a 24-hour scale, the changes in serum corticosterone thus appear to lead in phase those in gross body activity and almost certainly do not lag.

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[6] To be described elsewhere, constructed by K. Shimada with advice from Dr. E. Hartig and R. P. Featherstone.

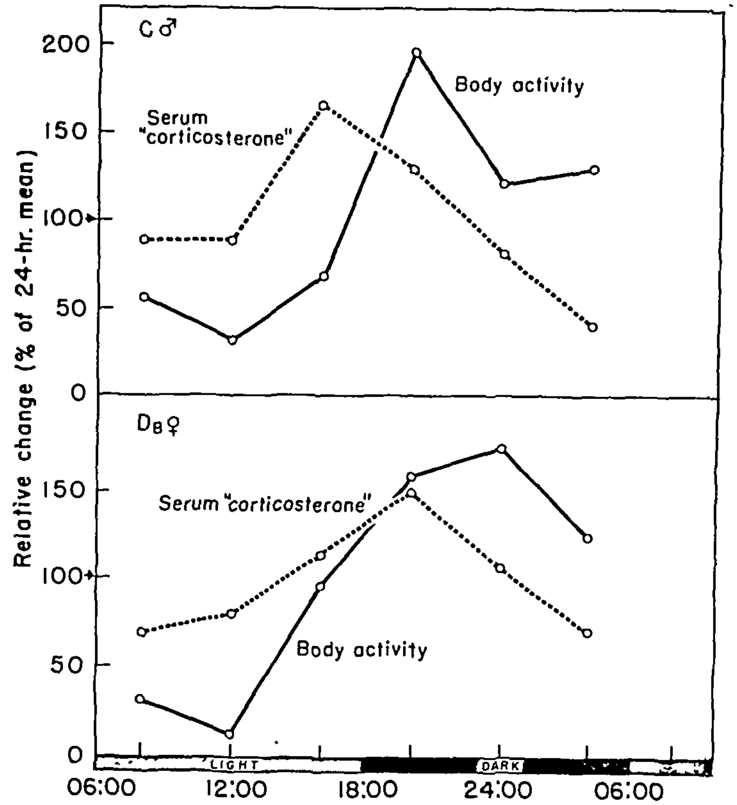

FIG. 3. Daily changes in gross body activity of two populations of mice[5] in relation to corresponding changes in serum corticosterone. The latter rhythm leads the former.

Reciprocal phase relations among 24-hour rhythms in blood eosinophils, serum corticosterone, and mitoses of adrenal cortical parenchyma may be examined in Figure 4. The top half of the figure shows that in the mouse a rise in eosinophils occurs before that in corticosterone, daily eosinopenia, in turn, lagging behind the start of corticosterone rise. Accordingly, the blood eosinophil peak occurs before that in corticosterone, while the peak of mitoses in adrenal cortex occurs later. The present, more complete curve for cortical mitoses agrees well with earlier results of spotchecks (10, 11). The relation of mitosis to secretion in the gland has previously been discussed on the basis of time relations between rhythms in eosinophils and cortical mitoses (10, 11); those earlier inferences seem to be supported by the phase relation of the curves for cortical mitoses on one hand and for serum corticosterone on the other. It is of further interest that the mitotic rhythm of the adrenal cortex is substantially out of phase with rhythms of mitoses in liver parenchyma or skin epidermis (29); the peak of the latter rhythms occurs during the middle of the light period, in animals of comparable stock and age, i.e., at a time when the adrenal cortical rhythm is below its day mean.

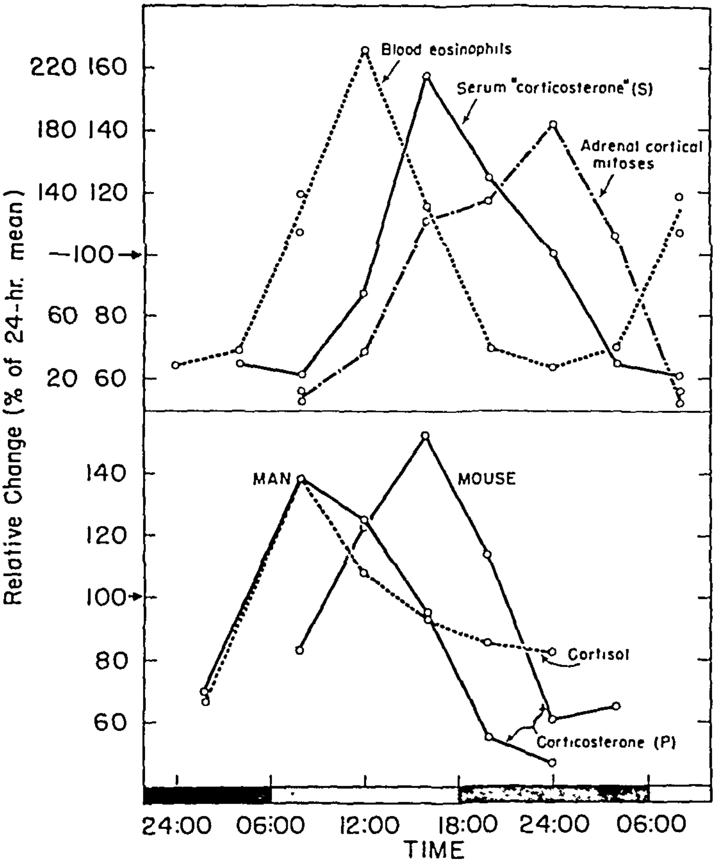

Fig. 4. Some parameters of the daily adrenal cycle. "Relative" values (% of 24-hour mean). The lighting regimen on abscissa applies strictly only to data on the mouse.

Lastly, a species difference in corticosterone rhythm between human beings on one hand, and mice on the other, is shown in the lower half of Figure 4. Data obtained by one of us on daily changes in blood cortisol and corticosterone of human beings (30) are graphed against the changes reported herein for serum corticosterone in the mouse. The curves of corticosterone in nocturnally active rodents and predominantly diurnal man are out of phase, thus revealing a difference in mode of synchronization with the environment.

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THE INFLUENCE OF CORTISONE GLYCINATE AND TESTOSTERONE GLYCINATE ON ADRENALECTOMIZED GOLDEN HAMSTERS AND CONTROL HAMSTERS

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ABSTRACT

The biological properties of cortisone glycinate and testosterone glycinate—two highly water soluble synthetic steroids—and of cortisone were compared in adrenalectomized and non adrenalectomized golden hamsters (Mesocricetus auratus). Special attention was paid to their effect on body and liver weight and liver components. Testosterone glycinate and also large doses of cortisone—1000 µg./100 gm. body weight—were found to have a toxic effect on golden hamsters whether adrenalectomized or not, while the toxicity of cortisone glycinate was very low. In contrast to testosterone glycinate cortisone and especially cortisone glycinate were effective in sustaining life in adrenalectomized animals. Cortisone caused an increase, testosterone glycinate a decrease in liver weight both in adrenalectomized and non adrenalectomized hamsters. With cortisone glycinate a normal development took place. The glycogenic properties of cortisone and of cortisone glycinate—but only after prolonged administration—were apparent both in adrenalectomized and in control hamsters, while testosterone glycinate did not posses any activity in this respect. The fat concentration of the liver was increased following administration of cortisone, but hardly responded to administration of both the other compounds. Cortisone and testosterone glycinate treatment of adrenalectomized animals caused a decrease in phospholipid concentration in the liver, whereas cortisone glycinate tended to maintain normal levels. Suggestions are presented in an attempt to explain these different effects.

THE biological properties of certain aminosteroids have been discussed in previous publications (1, 2, 3a, 3b, 4). Steroids linked with aminoacids were produced chiefly in order to increase the solubility of steroids. It was believed that—within the organism—these N-steroids would be rapidly hydrolysed to liberate the corresponding steroid. Subsequent experiments with such compounds showed that the biological action of the aminosteroids was not entirely identical with that of free steroids. Testosterone glycinate has no androgenic properties as compared with free testosterone; no testosterone glycinate was found unchanged in the urine (5). Even certain steroids linked with succinic acid (e.g. cortisone hemisuccinate) have biological properties different from those of free steroids (6). Jacoby and Tompkins (7) demonstrated, that hemisuccinates could be reduced without previous saponification.

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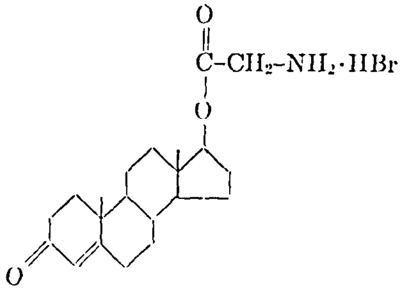
This paper presents the results of an investigation into the influence of two synthetic steroids—cortisone glycinate and testosterone glycinate (8)—on body weight, liver weight and liver composition in adrenalectomized golden hamsters (Mesocricetus auratus) and non-adrenalectomized controls.

METHODS

Experimental animals used where golden hamsters, as these are known to lack accessory adrenals (9, 10). The animals were separated according to sex and then divided into adrenalectomized groups and control groups, each group including at least 20 animals. The approximate body weight was 40–60 gm., and the approximate age 8–10 weeks. The animals were given a standard diet ad libitum and received daily injections of the N-steroid tested (200 μg./100 gm. body weight) and of cortisone (1000 μg./100 gm. body weight). Experiments were carried out over a period of 44 days; one group of adrenalectomized animals and one group of controls were sacrified for correlative analysis on the 7th, 14th, 24th, 34th and 44th day. In animals given steroids with a toxic effect or lacking life-sustaining properties, experiments naturally had to be terminated after 14 days or in same cases after 7 days.

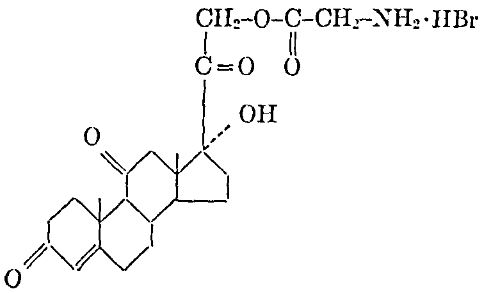
Plan of investigation

Experimental animals: golden hamsters (Mesocricetus auratus)
Divided into: adrenalectomized animals and controls
Treated with: cortisone (1000 μg./100 gm. body weight daily)
cortisone glycinate (200 μg./100 gm./day)
testosterone glycinate (200 μg./100 gm./day)
Interval until death: 7, 14, 24, 34, 44 days (animals sacrified by decapitation)
Factors determined:
- a) body
  1. initial and ultimate body weight
  2. ratio between body weight and liver weight
- b) liver
  1. liver weight
  2. nitrogen
  3. phosphorus
  4. glycogen
  5. fat
  6. phospholipids
  7. cholesterol
  8. bile esters



Testosterone glycinate hydrobromide

UVλ max alcohol	241 mu.
Molecular extinction alc.	14.700
UVλ max water	248 mu.
Molecular extinction water	14.100
Water solubility 200 mg./ml.	



Cortisone glycinate hydrobromide

UVλ max alcohol	238 mu.
Molecular extinction (alc.)	14.700
UVλ max water	245 mu.
Molecular extinction water	14.500
Water solubility 60 mg./ml.	

In alcohol, testosterone glycinate hydrobromide and cortisone glycinate hydrobromide have a UV absorption maximum of about 240 mu., which is characteristic of Δ^4,3-ketosteroids. Both are highly water soluble, in which respect they have a great advantage over cortisone or other steroids.

RESULTS

Normal untreated golden hamsters showed an increase in body weight up to 16% after 34 days; after adrenalectomy, animals showed a decrease in body weight of about 26%, and died after 5–7 days.

Testosterone glycinate was found to have a toxic effect on golden hamsters whether adrenalectomized or not. The majority died after 7 days, showing a slight decrease in body weight (about 8%). Testosterone glycinate proved to have no life-sustaining properties in the adrenalectomized animals, which died within 5–7 days after administration showing a decrease in body weight comparable to that in the control group.

Large doses of cortisone (1000 μg./100 gm. body weight) also had a toxic effect. The loss of weight accompanying administration of this substance has previously been reported by other authors. Prolonged administration of cortisone was found to be fatal. Most of the animals died after 20–30 days' treatment, showing a decrease in body weight of about 30%. Cortisone sustained life in adrenalectomized animals up to 34 days but failed to stop the loss of weight.

Cortisone glycinate did not affect normal development of body weight in golden hamsters during the first 24 days of administration. The increase in body weight throughout this period roughly corresponded with that in the controls. After prolonged treatment (exceeding four weeks) there was a very marked increase in body weight, up to 40%. This phenomenon was associated with an increase in liver glycogen concentration and premature maturation of reproductive organs. Cortisone glycinate was very effective in sustaining life in adrenalectomized animals, although there was a decrease in body weight during the first two postoperative weeks. The animals regained initial weight values and exceeded them, however, 4–5 weeks after adrenalectomy (Fig. 1).

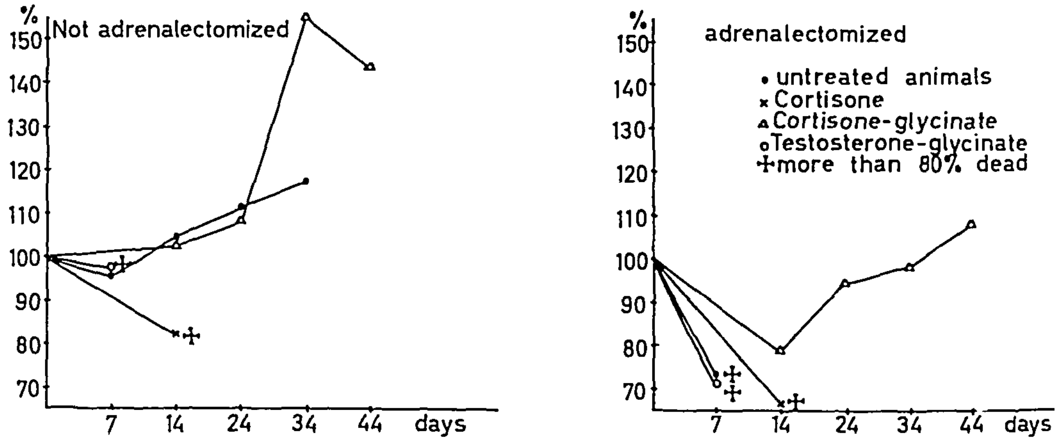
The influence of these synthetic steroids on the liver was investigated with special reference to their effect on glycogen, fat phospholipid, cholesterol, cholesterol-ester and phosphorus concentrations.

Findings showed that the average liver weight in normal golden hamsters was 3 gm. This was naturally reduced after adrenalectomy, chiefly due to loss of water and organic components. Liver weight was also reduced following administration of testosterone glycinate, both in adrenalectomized animals and in controls (Fig. 2).

Cortisone caused an increase in liver weight in adrenalectomized animals and controls. As a result of the decrease in body weight following administration of cortisone the percentual increase in liver weight relative to body weight was even more marked.

Cortisone glycinate slightly increased liver weight but only after prolonged administration (exceeding two weeks). Adrenalectomized animals treated with cortisone glycinate showed a decrease in liver weight within the first two weeks but regained initial weight values when administration was prolonged.

The ratio between body weight and liver weight is a useful aid in distinguishing between loss of body weight and loss of liver weight. In normal golden hamsters the ratio was found to be 6.4; a reduction of this ratio following adrenalectomy (down to 4.5) shows that the loss of liver weight exceeds the loss of body weight. It should be pointed out that all figures were statistically computed; the statistical significance of the deviation was also determined.



Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ
Not adr. ect.	7	20	95,3 ± 10,7			20	96,2 ± 15,4		
"	14	20	104,5 ± 12,7	10	102,5 ± 9,42			10	82,1 ± 25,4
"	24	20	111,1 ± 11,6	10	107,7 ± 10,8				
"	34	20	116,7 ± 10,0	10	155,2 ± 24,4				
"	44			10	143,5 ± 22,7				
adr. ect.	7	30	73,3 ± 15,66			40	71,8 ± 13,3		
"	14			10	78,9 ± 17,2			10	67,1 ± 16,9
"	24			10	94,6 ± 28,0				
"	34			20	98,1 ± 31,9				
"	44			20	108,1 ± 27,5				

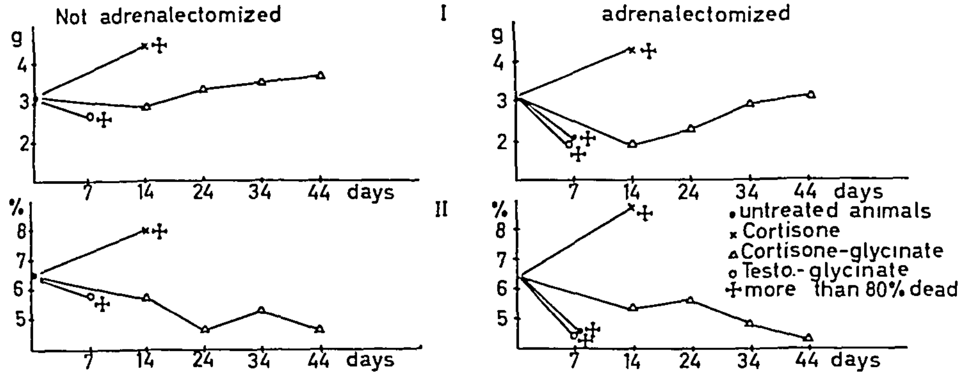
FIG. 1. Body weight curve in adrenalectomized golden hamsters and in controls. *Values represent percentage increase or decrease relative to initial weights.*

Liver glycogen concentration: this was about 13 mg. glycogen per gramme fresh liver weight in untreated controls—a figure corresponding with that found in other laboratory animals. The fall in glycogen concentration following adrenalectomy is attributable to glycogenolysis as a result of the operation, and to the lack of adrenal hormones which prevents the liver from forming new glycogen. Testosterone-glycinate had no glycogenic effect either in adrenalectomized or in control animals.

The glycogenic properties of cortisone glycinate were apparent both in adrenalectomized and in control hamsters but only after prolonged administration (exceeding 24 days). Cortisone had marked glycogenic properties event within a week of its administration and even in adrenalectomized animals (Fig. 3).

Overbeek *et al.* (11) investigated glycogen activity in starving rats and, under special conditions, established a limited glycogenic effect of cortisone glycinate. Cortisone hemisuccinate resembled glycinate in this respect.

The fat concentration in the liver in normal golden hamsters amounted to 30 mg./gm. liver weight. This concentration was increased following cortisone administration, and slightly decreased following administration of testosterone glycinate or cortisone glycinate.

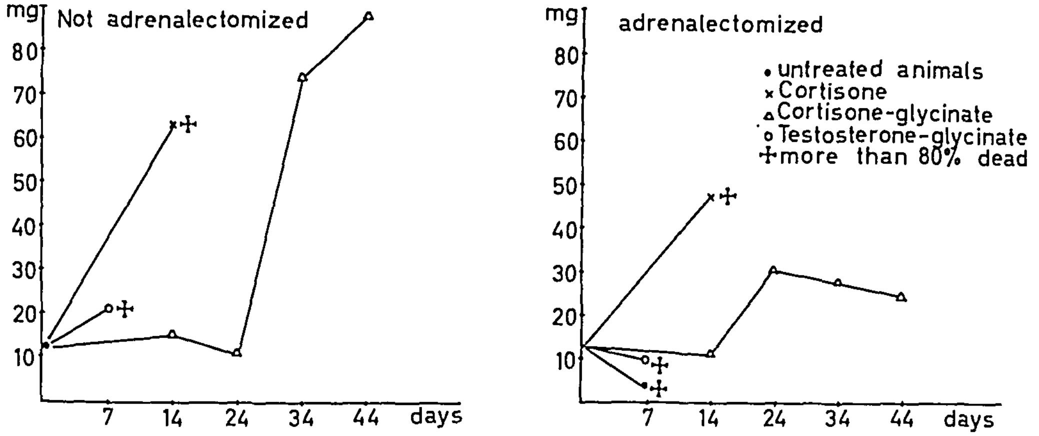


Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ
Not adr. ect.	7	28	3,13 ± 0,55			20	2,691 ± 0,64		
"	14			10	2,923 ± 1,41				4,456 ± 1,24
"	24			10	3,368 ± 0,99				
"	34			10	3,512 ± 1,31				
"	44			10	3,636 ± 1,07				
adr. ect.	7	30	2,096 ± 0,55			40	2,030 ± 0,57		
"	14			10	1,894 ± 0,92				4,337 ± 1,44
"	24			10	2,286 ± 0,98				
"	34			20	2,933 ± 1,42				
"	44			20	3,137 ± 1,40				

FIG. 2. Liver weight curve in adrenalectomized hamsters and in controls. The slope of the absolute values which are given in the table is shown in part I. Part II shows the values expressed by the ratio between liver weight and body weight.

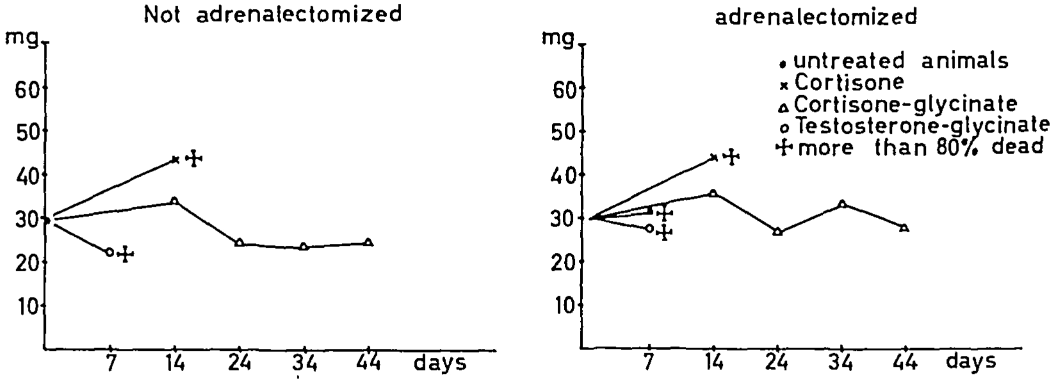
The liver fat concentration following adrenalectomy was 30 mg./gm. fresh liver weight—identical with the concentration prior to adrenalectomy in view of the decrease in liver weight which follows adrenalectomy, however, the total fat concentration of the liver was evidently reduced after the operation.

The fat concentration of the liver was increased following administration of cortisone, even in adrenalectomized animals, but it hardly responded to administration of testosterone glycinate or cortisone glycinate (Fig. 4). Kendall and Heilman (12) demonstrated that administration of several other steroids to mice was followed by a decrease in body weight



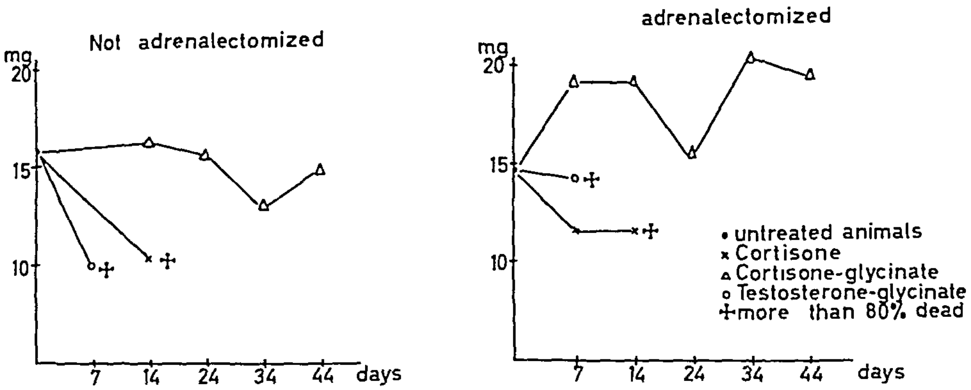
Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ	Animals	m ± 2 σ
Not adr. ect.	7	28	12,7 ± 4,36			20	21,0 ± 8,5		
"	14			10	15,1 ± 7,5			10	63,0 ± 42,8
"	24			10	11,0 ± 1,5				
"	34			10	74,0 ± 4,3				
"	44			10	81,3 ± 32,6				
adr. ect.	7	30	4,15 ± 4,04			40	10,1 ± 5,68		
"	14			10	11,4 ± 9,5			10	47,3 ± 40,8
"	24			10	31,7 ± 13,0				
"	34			20	27,5 ± 14,5				
"	44			20	24,9 ± 17,0				

FIG. 3. Liver glycogen curve in adrenalectomized hamsters and in controls. Values show absolute content in mg./gm. liver weight.



Treatment	Days	Controls		Cort.-glycinate		Testo.-glycinate		Cortisone	
		Animals	m ± 2σ	Animals	m ± 2 σ	Animals	m ± 2σ	Animals	m ± 2σ
Not adr.ect.	7	28	29,6 ± 7,04			20	22,0 ± 10,3		
"	14			10	34,1 ± 15,74			10	43,37 ± 14,4
"	24			10	24,5 ± 7,21				
"	34			10	23,4 ± 8,0				
"	44			10	23,9 ± 10,5				
adr.ect.	7	30	31,6 ± 7,29			40	27,9 ± 6,18		
"	14			10	36,2 ± 1,98			10	44,56 ± 12,4
"	24			10	27,1 ± 10,68				
"	34			20	33,0 ± 13,4				
"	44			20	27,6 ± 13,1				

FIG. 4. Liver fat curve in adrenalectomized hamsters and in controls. Values show absolute content in mg./gm. liver weight.



Treatment	Days	Controls		Cort.-glycinate		Testo-glycinate		Cortisone	
		Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ	Animals	m ± 2σ
Not adr. ect	7	28	15,8 ± 2,21			20	10,0 ± 4,59		
"	14			10	16,22 ± 9,41			10	10,44 ± 4,54
"	24			10	15,60 ± 5,48				
"	34			10	12,80 ± 2,64				
"	44			10	14,80 ± 5,8				
adr. ect.	7	30	14,8 ± 2,82	10	19,3 ± 10,05	40	14,3 ± 4,44	10	11,7 ± 5,37
"	14			10	19,2 ± 14,24			10	11,7 ± 2,85
"	24			10	15,2 ± 8,12				
"	34			20	20,5 ± 12,38				
"	44			20	19,5 ± 11,04				

FIG. 5. Liver phospholipid curve in adrenalectomized hamsters and in controls. Values show absolute content in mg./gm. liver weight.

and an increase in body fat (from 3 to 18%). Fatty degeneration of the liver has likewise been reported.

The phospholipid concentration of the liver in the animals under investigation amounted to about 50% of the fat concentration, the remaining 50% consisting of about 1/30 cholesterol, 1/50 cholesterol-ester and further neutral fat and fatty acids.

Administration of cortisone to adrenalectomized animals caused a decrease in phospholipid concentration per gramme liver weight. This was also the case with testosterone glycinate, whereas cortisone glycinate tended to maintain or to increase the phospholipid concentration (Fig. 5). An increase in cholesterol concentration per gramme liver weight was seen following adrenalectomy in the control group, whereas a decrease occurred following administration of testosterone glycinate and cortisone glycinate. The phosphorus level of the liver was determined on the basis of inorganic phosphorus. There was an increase during glycogenolytic processes (e.g. adrenalectomy), and only a slight increase during glycogenic processes (cortisone administration).

DISCUSSION

In the past few years there has been an increased interest in steroid-protein complexes (13–19) and in the steroid-amino-acid compounds (3a, 3b) as well as in the influence of some known steroids upon the liver composition (20–31). In an investigation of the question regarding the properties of such substances the use of synthetic crystalline compounds may be valuable.

Testosterone glycinate was found to have a toxic effect, whereas the toxicity of cortisone glycinate was very low in golden hamsters. Comparative investigations into larger doses of cortisone showed that cortisone is less toxic than testosterone glycinate but more toxic than cortisone glycinate. Cortisone glycinate was as effective as cortisone in life-sustaining experiments in adrenalectomized animals. Testosterone glycinate showed a complete lack of life-sustaining properties.

Both cortisone and testosterone glycinate caused a decrease in body weight, most marked in the case of cortisone although, as pointed out, testosterone glycinate was more toxic. Cortisone glycinate caused no decrease in body weight, normal development continuing until the 24th day. Weight development was accelerated after the 24th day, an increase to 40% being determined within 10 days. An accelerated increase in liver glycogen was likewise observed. It is difficult to explain this phenomenon. The golden hamster perhaps does not possess a specific enzyme to hydrolyse cortisone glycinate into free cortisone and glycine. Such an enzyme may be formed, however, after prolonged administration of cortisone glycinate. This theory would seem to be supported by the literature, which includes some reports on similar cases of adaptive enzyme formation. The change in the liver glycogen concentration following prolonged administration of cortisone glycinate shows a distribution curve identical with that of free cortisone within the first week of administration. Cortisone glycinate showed eosinophile depressing properties, as also in the case of cortisone. The problem becomes even more complicated when the body weight and fat composition of the liver are taken into account. Cortisone administration causes a decrease in body weight, whereas cortisone glycinate increases body weight. Only minor fluctuations in the liver fat concentration were seen following treatment of animals with testosterone glycinate or cortisone glycinate, whereas cortisone caused a rise in fat concentration, prolonged administration of large doses giving rise to fatty infiltration of the liver. The phospholipid content was decreased after cortisone or testosterone glycinate treatment, remaining within normal limits after cortisone glycinate administration. Amino-acid esters of steroids therefore would seem to have special biological properties in some respects. Certain other cortisone derivatives—e.g. cortisone hemisuccinate—showed antigenic properties when combined with bovine serum albumin in rabbits (32). Some testosterone derivatives with substitution in C_3 or C_{17} antagonize testosterone, e.g. testosterone-17-chlorocarbonate (33) and testosterone-3-(O-carboxylmethyl)-oxime (32). This corroborates our observation that cortisone glycinate showed a gonadotropic effect on golden hamsters, and it may be explained on the basis of a secondary effect. Schneider and Frahm (34) also described pituitary stimulation effected by some aminosteroids isolated from urine.

The biological properties of cortisone glycinate and some nitrogen-bearing steroids isolated from aqueous adrenal extracts require further investigation with a view to possibilities of practical application. Such compounds probably interfere with the fat metabolism, as suggested by Schmitz and Hilgetag (35) and Schmitz and Kühnau (36) with reference to certain adrenal fractions. The low toxicity and the biological properties in general, furthermore, render cortisone glycinate a suitable new synthetic compound for introduction into the series of applied hormonal preparations.

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LONG TERM EFFECTS OF DEUTERON IRRADIATION OF THE RAT PITUITARY[1]

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ABSTRACT

An investigation has been made of the long-term effect of low radiation doses to the rat pituitary using a high energy deuteron beam. The pituitaries were irradiated when the rats were 28 days old. The animals were maintained up to 27 months thereafter. A total of 138 controls and 169 treated rats were studied. Groups varying in size from 7 to 20 rats at each dose of radiation were sacrificed at intervals of about 3 months.

The endocrine changes became increasingly more prominent with increasing dosage and with time. Within certain dose ranges the same terminal results were obtained, though the lower the dose the longer the period which elapsed before the maximum effect resulted.

The effect of the radiation on pituitary structure was as follows: decrease in size of all lobes; loss of demarcation between lobes; increased connective tissue in surrounding membranes and supporting framework and frequent small hemorrhages. In the anterior lobe the change effected in the shortest time and at the lowest dose was degranulation and disappearance of acidophils. Basophils were less severely damaged. This differential action of radiation on the two types of chromophils was not noted in the earlier series of experiments reported, where higher deuteron dosage was applied to the pituitary and prompt injurious action on its parenchyma resulted.

The endocrine changes in organs dependent on pituitary secretion appeared in the following sequence: reduction in adrenal weight with adrenal cortical atrophy; decrease in thyroid size and evidences of inactivity; testicular atrophy, regression of male accessory organs and failure of sperm production. The sensitivity of body growth impairment was difficult to evaluate relative to other endocrine alterations, at least at lower doses of radiation, after which the endocrine changes did not supervene at once; body size, particularly length, could not regress, whereas the weight of the endocrine organs could. The severity of all these changes following irradiation, judged by weight or histological structure or function, increased with dosage as did the speed of onset.

Adenomata were observed in pituitaries which had been irradiated with low doses. At the lowest doses, tumor incidence in irradiated pituitaries was three fold greater than in pituitaries of controls of the same age, whereas, at high doses tumor formation was suppressed. The tumors were derived from chromophobes, chiefly, but from acidophils and basophils as well; mixed-cell adenomata were also observed.

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[1] Aided by a grant from the American Cancer Society, California Division, ACS 150, 1955–57 and a contract between the AEC and the University of California, Berkeley.

Tumors occurred rather frequently in other endocrine organs during the second year. They were no more frequent in experimental than in control animals, and after high radiation dosage no adenomata were present in the adrenal cortices or thyroids which had become hypoplastic as a result of pituitary injury.

IT HAS been shown that the pituitary of the rat can be completely or partially destroyed by a well-localized beam of high energy deuteron particles, without evidence of serious damage to adjacent structures (1). In the first series of experiments in which such radiation was directed to the rat pituitary, massive doses were given, ranging from 3000 to 25,000 rep.[2] The observations made on groups of rats sacrificed at intervals from 4 days to 9 months post-radiation led to the following conclusions:

1. The degree of destruction of the pituitary following radiation is a function of the post-irradiation period as well as of the dose.
2. Only the highest doses, 18,900 rep and above, led to prompt and complete destruction of the pituitary. After lower doses, 6300 rep or less, the destruction was not complete even many months after irradiation (9 months).
3. There was no evidence of stimulation of the pituitary after the doses and periods of observation employed, either from morphology of the anterior pituitary or from function judged by the dependent endocrine organs.
4. There was no evidence from the dose levels employed that the pituitary recovered after the damage caused by radiation at dose levels employed. Although mitoses were observed occasionally they were abnormal.
5. No difference in sensitivity of pituitary cell types was reported, the high doses used being equally destructive to all.
6. Body weight increases and thyroid weights were impaired soon after irradiation with dose levels from 6300 to 18,900 rep, but degeneration of the testes occurred much later.

The present study is an extension of the previous work to include lower doses of deuteron radiation and longer post-irradiation intervals. The specific objectives were as follows: to search further for evidence of a differential effect of pituitary irradiation on cell types, particularly those of the anterior lobe; to determine the long-term destructive effects of low doses on anterior lobe morphology and function, and to observe any increase in the incidence of tumors in the pituitary or in other tissues of the body.

MATERIAL AND METHODS

The procedure of alignment of the pituitary in the path of the deuteron beam, the size of the aperture, the roentgenographic check of alignment, and the method of measurement of radiation dose and its spread have been explained in detail in the previous publication (1). The deuteron beam was collimated to conform to the shape and dimensions of the pituitary by being passed through a brass aperture of triangular shape slightly larger than the pituitary. Having once determined roentgenographically with the aid of a lead phantom placed in the pituitary site in relation to the occipitosphenoidal synchondrosis, it was possible to direct the deuteron beam correctly by relating it to this landmark.

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[2] To conform with the previous publication in this series, doses are reported in rep. The new international dose unit is rad; 1 rad = 1.06 rep. = 100 erg per gm. tissue

A group of 169 male rats of the Long-Evans strain were subjected to pituitary irradiation at 28 days of age with doses of 945, 1575, 3150 and 6300 rep. The normal controls (138 rats) were the same age and were maintained under the same conditions. As in the previous series all rats were given a complete diet which was fed *ad libitum.* Periodically the general appearance, muscle tone, body weight, tail length and any grossly evident abnormalities were recorded. Groups composed of 7 to 20 rats which had received each dosage were sacrificed 3, 7, 12, 17, 22 and 27 months after irradiation. The testes, seminal vesicles, prostate, adrenals, thyroid, thymus and pituitary were dissected, weighed and fixed for histologic study. The testes and thyroid were fixed in Bouin's fluid and embedded in paraffin and stained with hematoxylin and eosin. The adrenals were fixed in neutral formalin and sectioned while frozen to be stained, subsequently, with Sudan black for lipid. The pituitary glands were fixed in Zenker-formol, sectioned at 4 micra after nitrocellulose embedding and stained with a modified Mallory-Azan stain (2) and also according to the periodic acid Schiff (PAS) technique following Purves and Griesbach (3). Sections from 5 levels of the pituitary were studied.

RESULTS OF PITUITARY IRRADIATION ON STRUCTURE AND FUNCTION OF ENDOCRINE GLANDS

The effects of irradiation of the pituitary were estimated both by assessment of its functional state during the life of the animal and by the morphological changes in the dependent endocrine organs, as well as by direct analysis of morphological changes in the pituitary. Changes in growth hormone secretion were estimated from measurements of gain in body weight and dimensions. The effect on skeletal growth, as indicated by the average increase in tail length, is shown in Figure 1. (Data from the preceding series is included for purposes of comparison.) The increase in dimensions was not affected by 945 rep, but at dose levels of 1575 rep and above, tail growth began to lag behind that in normal rats, the retardation occurring sooner and being progressively greater at higher doses.

In order to evaluate the dose-effect relationship the data were analyzed as follows. It was assumed that that part of the increase in tail length which is dependent on pituitary function is the difference between the tail length of normal rats and that of surgically hypophysectomized animals. The tail length increase, 22 months post irradiation, of groups receiving the various doses, is plotted as per cent of normal in Figure 3. It can be seen from the figure that pituitary irradiation resulted in an impairment of tail growth increment which was exponential.

Let:

l_D = tail length increase of radiated rats receiving dose D
l_h = tail length increase of surgically hypophysectomized controls
l_n = tail length increase of normal rats
D_{50} = dose necessary to reduce to 50% the pituitary dependent tail growth (difference between tail length of intact and hypophysectomized controls)
e = base of the natural system of logarithms

Then the percent change in tail increment is as follows:

$$\phi_T = 100\,\frac{l_D - l_h}{l_n - l_h} = 100e^{-0.693D/D_{50}} \qquad D_{50} \approx 5{,}500 \text{ rep}$$

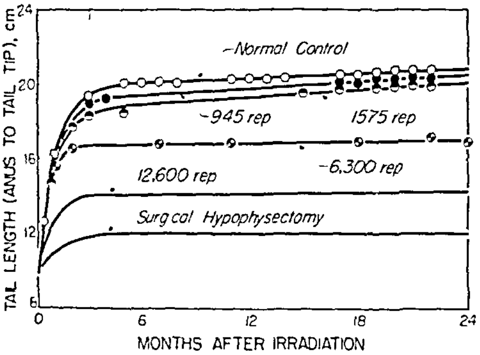


Fig. 1. Effect of pituitary deuteron irradiation on tail length of male rats. (Results from dose of 12,600 rep are interpolated (*1*).)

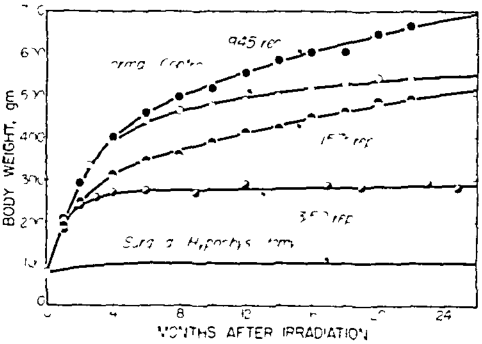


Fig. 2. Body weight of rats following various doses of pituitary irradiation. After 945 rep a portion of the rats became obese *during the second year.*

The average increases in body weight after pituitary irradiation as a function of time are plotted in Figure 2. Doses of 1575 rep resulted in slowing of body weight increase but not in stasis. At 3150 rep marked decrease in rate of weight gain occurred within 1 month. The higher the dose the more rapidly stasis followed, but at no dose did growth stasis follow as promptly as after surgical hypophysectomy.

When M_D is the body mass of rats having received a dose D, M_h the mass of hypophysectomized *rats, and* M_n *the mass of normal controls, then:*

$$\phi_m = 100\,\frac{M_D - M_h}{M_n - M_h} \qquad D_{50} \approx 5500 \text{ rep.}$$

Comparison of ϕ_t and ϕ_m in Figure 3 indicates the essential similarity of action of pituitary irradiation on tail length and body mass, when doses above 3000 rep were given. The dose required for reduction of post-irradiation growth to half normal is about 5500 rep (D_{50}) by each criterion, and the dose-effect relationship is a negative exponential.

At the lowest dose, 945 rep, the body weight failed to reach a plateau.

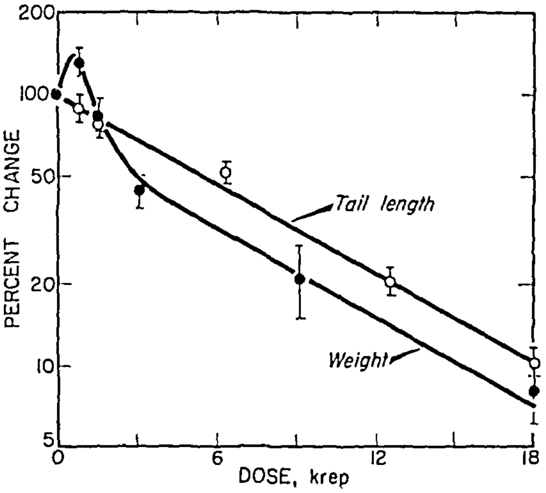


FIG. 3. Dose dependence of tail length and body weight (ϕ_l and ϕ_m) as function of deuteron dose 22 months after irradiation. Note the increase of body weight due to fat gain after the lowest dose.

but continued to increase with time. This lowest dose not only did not retard skeletal or body weight increase; instead, body weight eventually exceeded normal due to accumulation of fat. The development of obesity was noted during the first year but became definite in the 2nd year. The late appearance of adiposity did not occur in all rats of the group; 8 of 12 weighed above the normal range. The heaviest rat attained a weight of 8.00 gm., a weight 300 gm. greater than the average of controls.

In order to obtain evidence of progressive change in the organs under control of the pituitary, the status was evaluated by the weight and morphology at increasing times following pituitary irradiation. The average weights of the thyroid, adrenals, prostate, seminal vesicles and testes at various post-irradiation intervals are shown in Figures 4, 5, 7, 8 and 9.

The order in which the different endocrine organs reflected pituitary damage was the same, whether judged by the time interval before reduction of activity became manifest, or by the size of the dose necessary to produce atrophy within a given interval. The lowest dose, 945 rep, caused reduction only in adrenal weight; at 1575 rep the weight of the thyroid

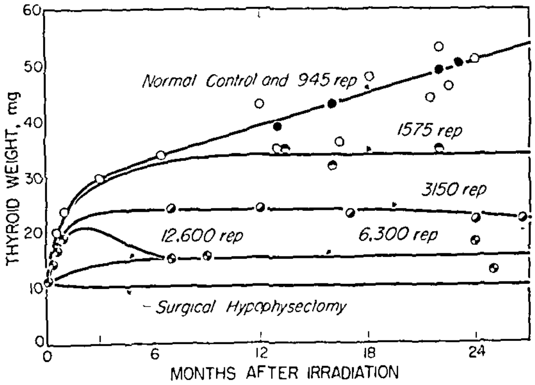


FIG. 4. Thyroid weights following pituitary deuteron irradiation.

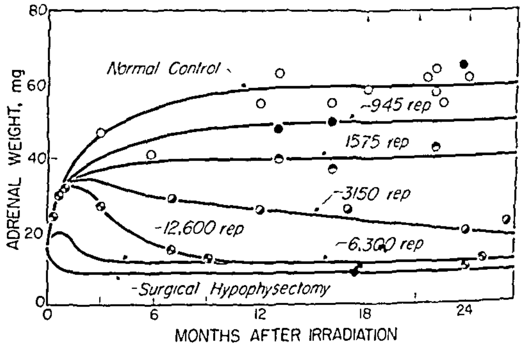


FIG. 5. Adrenal weight following pituitary deuteron irradiation.

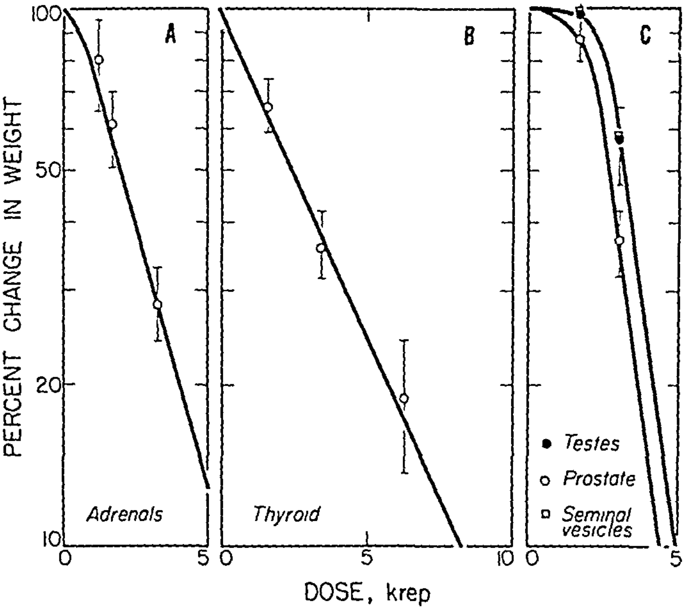


FIG. 6. A. Dose dependence of adrenal weight ($\phi_{ad.}$) 18 months after pituitary deuteron irradiation. B. Dose dependence of thyroid weight ($\phi_{th.}$) 18 months after pituitary irradiation. C. Dose dependence in weight of prostate, seminal vesicles and testes ($\phi_{pr.}$ ϕ_{sv} $\phi_{te.}$). 18 months following pituitary irradiation.

as well as of the adrenal was reduced; at 3150 the weights of all endocrine organs were reduced. Microscopic examination revealed a degree of aplasia comparable to the reduction in weight at the higher doses, eventually being comparable to the atrophy after complete surgical hypophysectomy. The order of decreasing sensitivity of the pituitary target organs to pituitary damage was therefore adrenal, thyroid, testis (seminal vesicles and prostate).

Normal function, even growth and differentiation of the pituitary target organs progressed for variable periods after irradiation, the time intervals before regression being dependent on the size of the dose given. At lower doses, 945 and 1575 rep, the testis descended into the scrotum, enlarged,

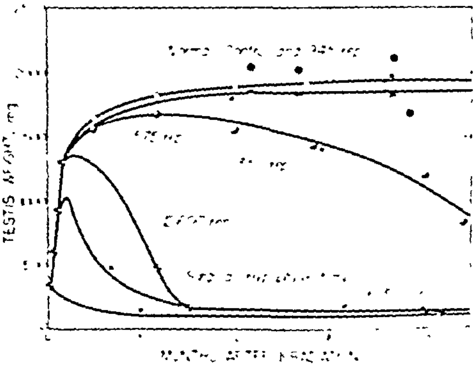


FIG. 7. Testis weight following pituitary irradiation.

and differentiated with normal sperm formation; at these low doses sperm were still present 2 years after irradiation. After 3150 rep the testes of half the animals had withdrawn from the scrotum within 2 years and resembled those after surgical hypophysectomy. After radiation with 6300 rep, half the testes were intra-abdominal by 10 months and all were atrophic by 2 years. Atrophy followed much more quickly after 12,600 rep; the testes of half the animals had withdrawn from the scrotum by 3 months. Testes appear to be the most sensitive of any target organ to small amounts of surviving pituitary tissue after radiation. The testis is also the most sensitive to minute fragments of pituitary left after surgical hypophysectomy.

The relative masses of thyroid and adrenals eighteen months after radiation, with respect to those of surgically hypophysectomized rats, are plotted in Figure 6, A and B.

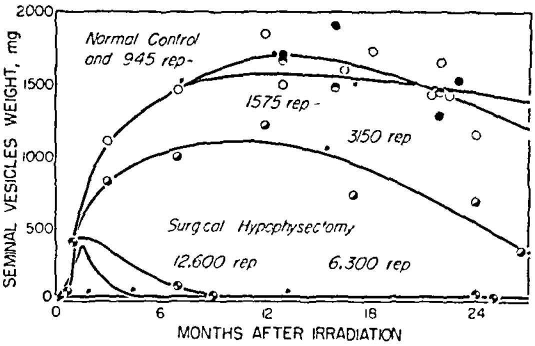


FIG. 8. Seminal vesicle weight following pituitary irradiation.

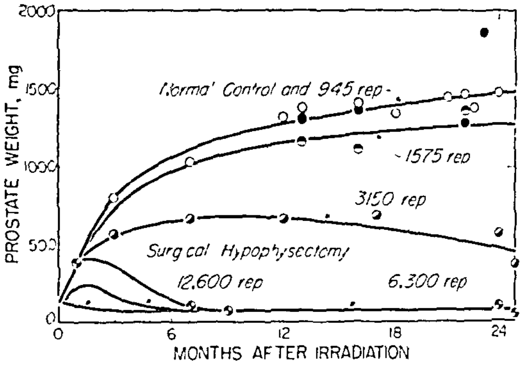


FIG. 9. Prostate weight following pituitary irradiation.

It appears that the adrenals and thyroid were much more affected by pituitary irradiation than is body weight; $D_{50\ \mathrm{Adrenal}} \approx 2000$ rep and $D_{50\ \mathrm{Thyroid}} \approx 2500$ rep.

At 18 or 22 months post-irradiation there was, with the exception of the lowest level, an exponential dose-effect relationship, resembling a "one hit" type radiobiological response for body mass and mass of thyroid and adrenals. However, the testes, prostate and seminal vesicles showed a different relationship. The absolute weights of testes, prostate and seminal vesicles are shown after different doses and time intervals in Figures 7, 8 and 9; the relative decrease is shown in Figure 6 C at 18–22 months; the response curve is of the "many hit" type. For the testes and dependent organs 50% normal weight was reached at approximately 3000 to 3500 rep.

MORPHOLOGICAL CHANGES IN THE PITUITARY

The weights of the pituitaries were markedly reduced after all dose levels, and the degree of atrophy progressed with dose (Table 1). After irradiation with 945 rep the pituitary weight was reduced to half normal by the end of the first year; a dose of 1575 rep resulted in a pituitary weight one-third normal by this time; after a dose of 3150 rep the pituitary weight was reduced to less than a third by 3 months; following irradiation with 6300 rep the pituitaries were a tenth the weight of pituitaries of normal rats by the 6th month, and no further reduction in weight occurred during a longer experimental period. The weight of the irradiated pituitary is not necessarily representative of its functional capacity, since different cell types may be damaged to a different degree. Pituitaries weighing 1–2 mg. were composed largely of a framework of connective tissue, sometimes augmented by scarring and the residue of hemorrhages.

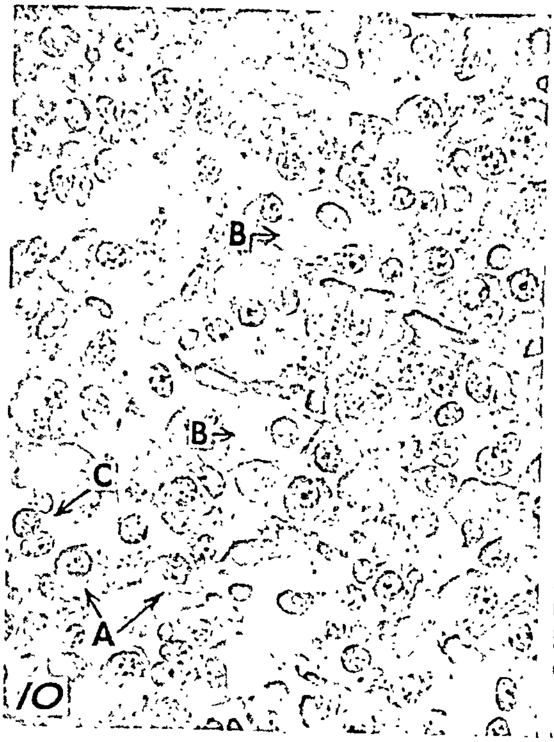
The pituitaries which were severely damaged by high doses (3150 and 6300 rep) were flattened, translucent and pale, but occasionally were hyperemic. The covering membrane was thickened and microscopic examination showed considerable scarring and heavy strands of connective tissue penetrating the parenchyma. A difficulty experienced on gross examination at autopsy in distinguishing individual lobes proved to be due to irregular invasion of cells from one lobe into neighboring lobes. After the lower doses (945 and 1575 rep) these changes were not so evident though other gross abnormalities such as tumors, cysts, and generalized hemorrhages were more frequent. None of the latter abnormalities were observed during study of 79 pituitaries irradiated with 3150 or 6300 rep whereas 16 of 39 pituitaries showed these changes after 945 rep and 10 of 35 pituitaries showed them after 1575 rep; they appeared in only 7 of the 129 control pituitaries.

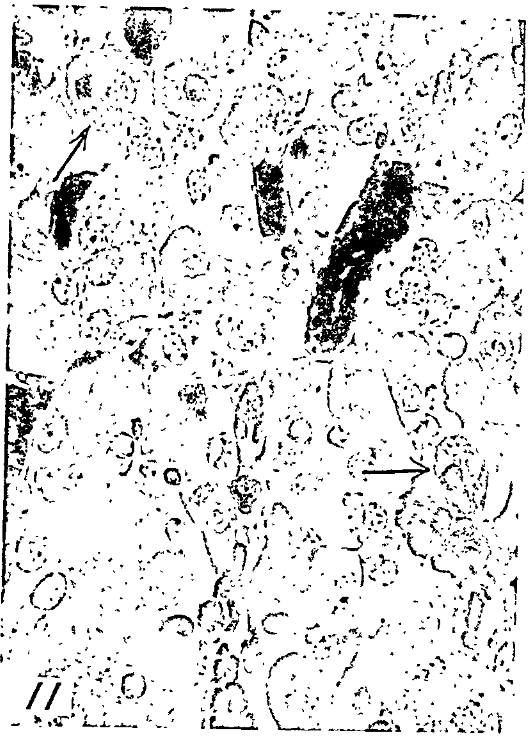
TABLE 1. PITUITARY WEIGHTS* AFTER IRRADIATION OF THE PITUITARY WITH INCREASING DOSAGE, AFTER PROGRESSIVELY LONGER PERIODS

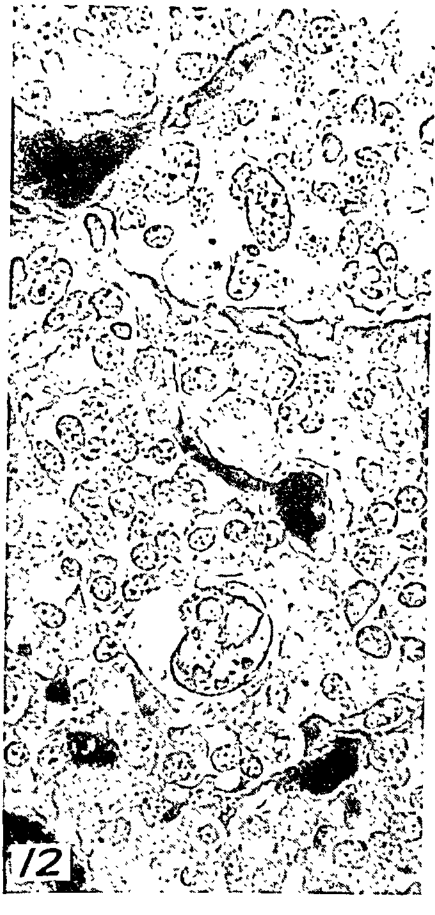
Dosage rep	Period after radiation in months					
	3–4	6–9	11–14	15–19	20–23	24–27
	mg.	mg.	mg.	mg.	mg.	mg.
0	10.2	10.7	12.0	12.1	12.4	13.3
945	—	—	5.9	6.5	8.4	—
1575	—	—	3.9	3.8	4.1	—
3150	2.8	1.9	1.4	1.8	1.3	1.4
6300	—	1.2	1.4	—	—	0.9

* Pituitaries with grossly discernible abnormalities were eliminated in preparing these averages.

The microscopic alterations in the parenchyma of the anterior lobe after the highest dose, 6300 rep, were advanced by 6 months. Normal acidophils had practically disappeared from the anterior lobe; those which could still be distinguished were almost completely degranulated or were atypical in size and nuclear morphology. The basophils, although reduced in number and partially degranulated, were less damaged; small groups could still be found in about half the glands confined to the gonadotrophic basophil zone. Such basophils were often abnormal, and showed vacuolation either of the type present after castration or after thyroidectomy. In the intermediate lobe the chief changes were loss of the usual uniform distribution of cells and the great variability in cell size and staining reaction. The posterior lobe was the least affected; however, considerable irregularity







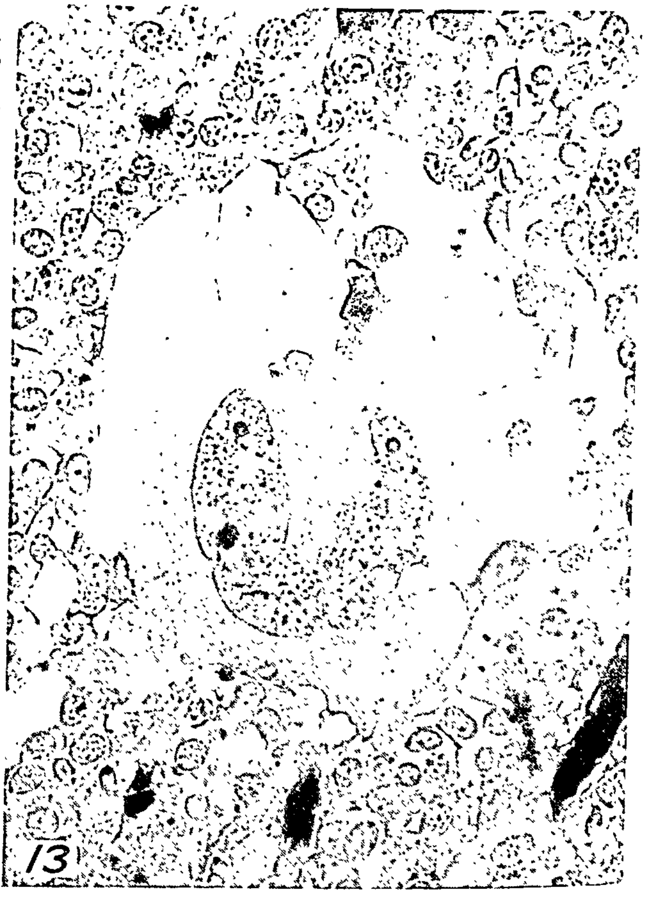


FIG. 10. Anterior pituitary of 13 mo. old control, showing normal appearance and distribution of (A) acidophils; (B) basophils; and (C) chromophobes. Mallory-Azan stain, ×500.

in cytoplasmic and nuclear size and shape was present (Fig. 17). Small localized hemorrhages were observed in all lobes. All the changes described were marked by 6 months, and maximal by 9 months; no further degeneration and no evidence of regeneration was detected up to 2 years post-irradiation.

After irradiation with 3150 rep the acidophils could still be recognized after 3 months. In the period from 6 to 24 months the changes, particularly in the acidophils, were progressive and by the end of this period many glands were indistinguishable from those which had received the higher dosage. In some glands the basophils were not decreased in numbers or *extensively degranulated, regardless of the length of post-irradiation* period, but had come to resemble closely the basophils of rat pituitaries after gonadectomy or after thyroidectomy. The changes in the intermediate or posterior lobes were similar to those observed after higher doses.

The pituitaries subjected to 1575 rep showed similar morphological changes but they appeared more slowly; the changes in acidophils found after 12 months were similar to those recorded for glands 3 months following 3150 rep. Basophils could be found throughout the majority of the glands; many were enlarged and showed nuclear changes and cytoplasmic vacuolation resembling those after castration or thyroidectomy (Figs. 10 and 11). Pituitaries irradiated with this dose showed no further morphological changes after 18 to 24 months, except that the acidophils virtually disappeared. Mitoses were increased at this dose and occurred in all lobes and in all cell types including chromophobes. The changes in the intermediate and posterior lobes were similar to those after 31.50 rep.

After irradiation of the pituitary with 945 rep the parenchymal elements in all pituitary lobes showed the same type of alterations as previously observed but the changes appeared more slowly. The acidophils were decreased in number and degranulated although to a lesser degree than in the preceding groups. The number of basophils remained within normal limits or appeared to be increased. Many of the basophils were abnormal showing various types of cytoplasmic vacuolation, pyknotic nuclei and peripheral clumping of granular material.

←

FIG. 11. Anterior pituitary 13 mo. following irradiation with 1575 rep. Acidophils had virtually disappeared whereas basophils were increased considerably in number and size and had a prominent Golgi apparatus. Some pyknosis of nuclei, and various types of cytoplasmic vacuolation were present (arrows). M.-A., ×500.

FIG. 12 and 13. Anterior pituitary 13 and 15 mo. following irradiation with 945 rep. showing pre-adenomatous changes. Clusters of atypical chromophobes, which sometimes reached gigantic proportions, were present. Note that acidophils were virtually absent. M.-A., ×500.

INCIDENCE OF TUMORS

Adenomatous Changes in Pituitaries

Adenomatous transformation occurred frequently in the pituitaries of the normal rats during the second year of the experiment. Gross abnormalities were noted at autopsy in 6 of the 116 control pituitaries examined.

TABLE 2. INCIDENCE OF TUMORS IN ENDOCRINE ORGANS DURING SECOND YEAR AFTER IRRADIATION OF THE PITUITARY

	Dosage rep	Total number of rats	Incidence of tumors		Conclusion
			No. of rats	Per cent	
Pituitary		116	37	32	No adenomas at high levels; at low levels more frequent than in controls and number per gland greater
	945	39	37	95	
	1575	32	21	65	
	3150	37	1	7	
	6300	6	0	0	
Adrenal cortex		125	35	28	Small, frequently multiple, tumors in controls and in experimental rats, except after high doses
	945	39	9	23	
	1575	41	5	10	
	3150	51	10	20	
	6300	12	1	8	
Thyroid		125	33	26	Multiple adenomas were frequent, except in glands where hypoplasia from high radiation dosage had occurred
	945	39	15	40	
	1575	41	7	16	
	3150	51	14	27	
	6300	12	2	17	
Adrenal medulla		125	10	10	Adenomas rare in all groups; none observed after high levels of radiation
	945	39	2	5	
	1575	41	1	2	
	3150	51	0	0	
	6300	12	0	0	
Parathyroid		125	5	4	Incidence low and no increase after radiation
	945	39	0	0	
	1575	41	0	0	
	3150	51	2	4	
	6300	12	1	8	
Reproductive system		125	2	2	No increase in incidence from radiation
	945	39	0	0	
	1575	41	0	0	
	3150	51	1	2	
	6300	12	0	0	

Tumors were first observed after maintenance for 16 months, and pituitary tumors were found histologically in 32% of all controls sacrificed between 16 and 24 months (Table 2). In glands which had been subjected to the higher doses of deuteron radiation, 3150 to 6300 rep, and in which severe damage to the parenchyma occurred promptly, no gross abnormalities were noted on removal of the gland and histological evidence of tumor formation was observed in only one gland. In the groups receiving the lower doses of radiation gross abnormalities were noted at autopsy in 12 of 39 pituitaries in the group irradiated with 945 rep and in 4 of the 32 irradiated with 1575 rep. The total number of tumors and the rate of appearance were both greater in the groups receiving these two lowest levels, than in controls; after 1575 rep tumors appeared by 13 months and the incidence rose with time until between 16 and 24 months more than half (65%) of the pituitaries had developed tumors. Following 945 rep adenomatous changes were observed in 75% of the animals sacrificed after 13 months, and by the end of the second year adenomatous transformations had developed in almost all the pituitaries (95%). The tumors in the irradiated pituitaries were frequently multiple, more so than in controls, 3.1 were found per gland by 24 months compared with 1.6 in controls (Table 3).

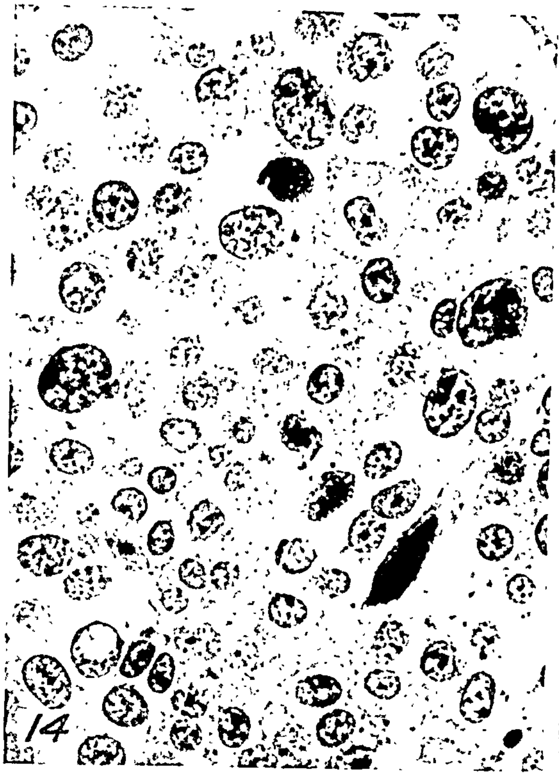
The adenomatous changes occurred most frequently in the anterior lobe. The tumors were usually microscopic, less frequently macroscopic. Pituitaries enlarged by such tumors attained weights of 40 to 327 mg. and dimensions up to 11×7×7 mm. Most of the tumors were clearly outlined, though some were poorly defined. Some were hyperemic, or even hemorrhagic. The large number of macrophages containing pigments derived from hemoglobin bore witness to the long period during which hemorrhages had been occurring.

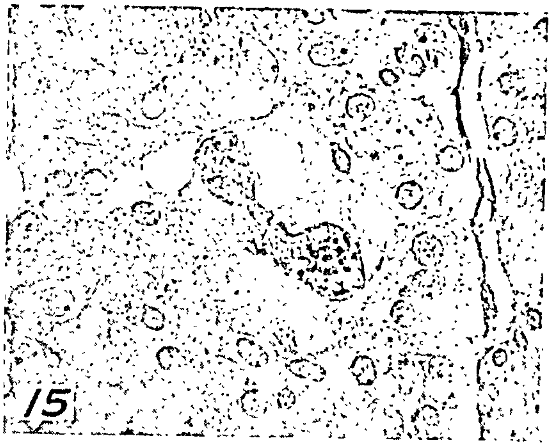
TABLE 3. CELLS OF ORIGIN OF TUMORS PRESENT IN PITUITARIES DURING SECOND YEAR AFTER IRRADIATION

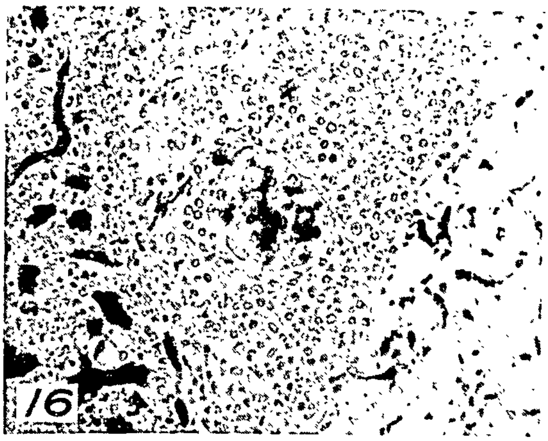
Dosage	No. of glands having tumors	Anterior lobe				Intermediate lobe cells	Tumors	
		Acidophils	Basophils	Chromophobes	Chromophobes & acidophils		Total No.	Av. per gland
	37	1	27	30	1	0	59	1.6
945	37	16	29	50	14	6	115	3.1
1575	21	13	7	30	4	5	59	2.8
3150	1	0	0	1	0	0	1	1.0
6300	0	0	0	0	0	0	0	0

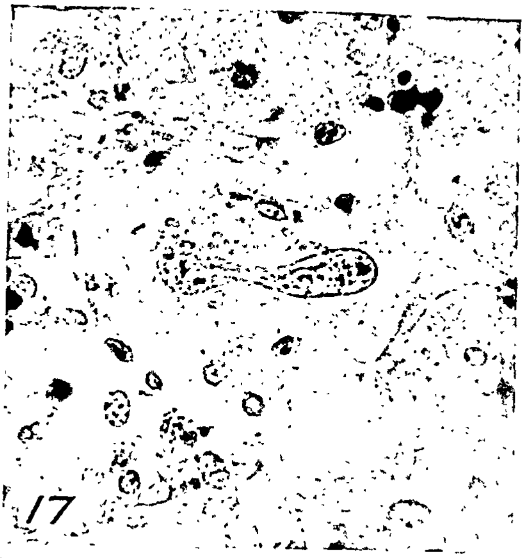
Adenomata derived from each of the three cell types were present, also tumors derived from mixed cell types (Table 3). The most dramatic increase took place in the acidophilic and in mixed acidophilic and chromophobic tumor classes; the former increased 16 fold in the 945 rep group. The incidence of tumors derived from basophils or chromophobes did not increase so markedly above that in controls. Intermediate lobe tumors appeared in rats irradiated with low doses whereas such tumors were absent in the controls.

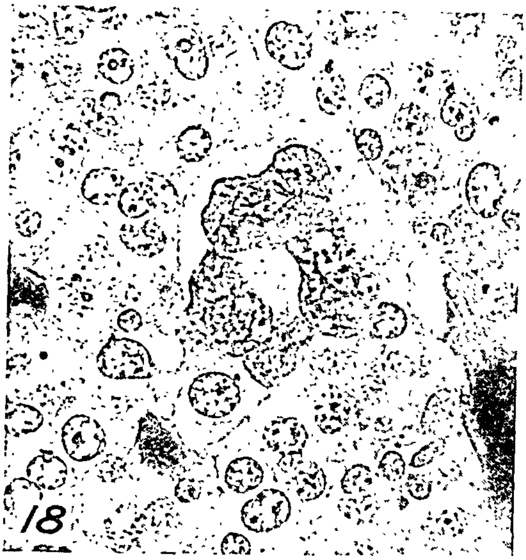
The cells of the adenomatous areas tended to be uniform in size and frequently smaller than the corresponding normal cell type. In some the cells were larger than normal, in which case the shape and inner structure of both cytoplasm and nucleus were frequently distorted, and the staining characteristics of the cytoplasm changed, due both to depletion of the











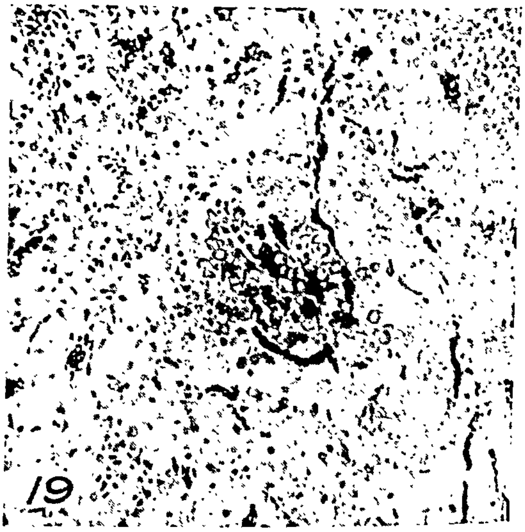


Fig. 14. Pre-adenomatous changes in the intermediate lobe, 23 mo. following irradiation with 1575 rep. Note considerable variations in cellular size and pronounced nuclear changes. M.-A., ×500.

granular content and to vacuolation of the cytoplasm. The number of mitotic divisions was notably increased in the adenomata and mitotic figures were often abnormal.

Tumors derived from chromophobes were the largest, as well as most common, and they were the only tumors in which hemorrhages were observed. Mitotic divisions were also more frequent in the chromophobic tumors. The cells did not stain intensely, and were usually not clearly outlined. In a few tumors the cells were irregularly enlarged; some attained gigantic dimensions. The outlines of the nuclei in these large cells were distorted by budding or folding of the nuclear membranes (Fig. 18) and nucleoli were prominent. The cytoplasmic outlines were more clearly defined than in the usual chromophobe.

Small PAS-positive granules were present in some of the tumors designated as chromophobe tumors, suggesting that these tumors might more properly have been grouped with basophilic tumors. Such tumors were usually smaller and less definitely outlined, and no hemorrhagic areas were present. The outlines of the cells designated here as "basophilic chromophobes" were more clearly defined than in typical chromophobic tumors. The cell size was usually smaller than that in normal basophils, and the cytoplasm stained purple rather than blue with Mallory-Azan. The nuclei were compact and polychromatic and the negative image of the Golgi apparatus was smaller than in normal basophils. A small number of the tumors was composed of cells clearly identifiable as basophils (Figs. 22 and 23).

It was also possible on the basis of staining criteria to identify some of the chromophobic tumors as composed of "acidophilic chromophobes." Tumors composed of clearly defined acidophils were the least common. Such tumors were usually small, and though not sharply outlined, were distinguishable with Mallory-Azan stain from the surrounding parenchyma by the presence of deep red or brilliant yellow stained granules (Figs. 19

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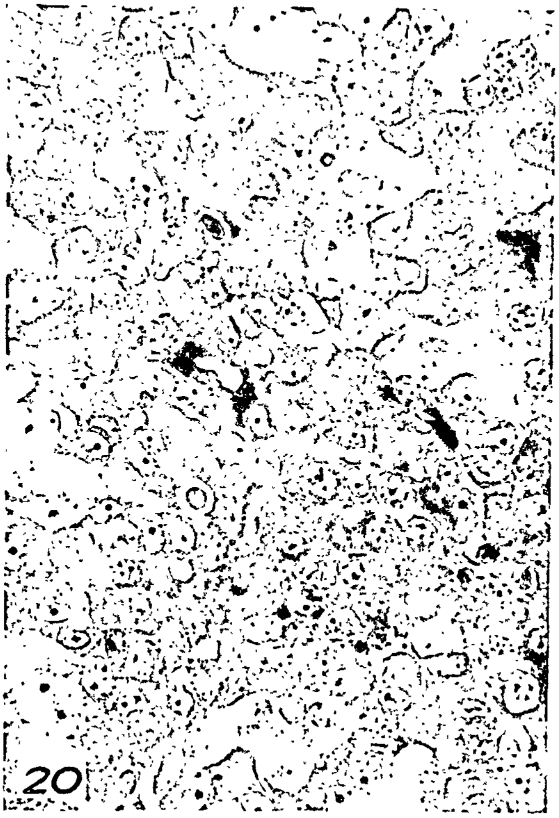
FIG. 15. Gigantic cell with atypical nucleus in pre-adenomatous area of the intermediate lobe, 13 mo. following 945 rep. M.-A., ×500.

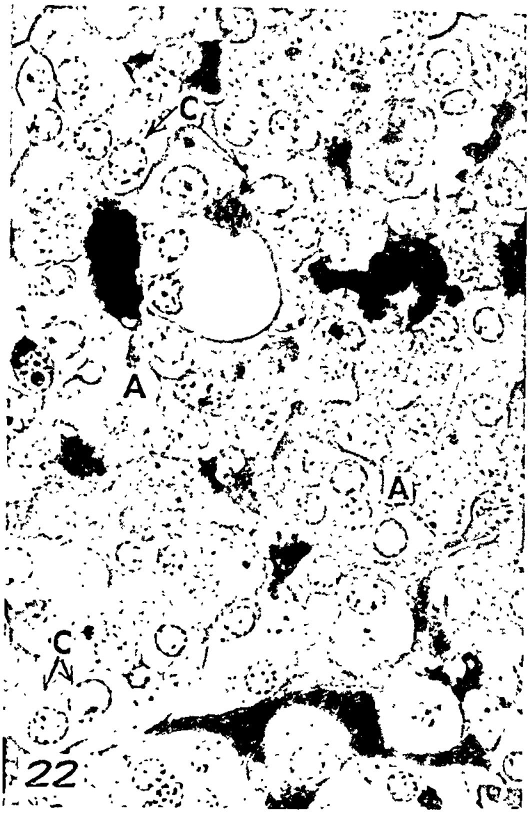
FIG. 16. Small intermediate lobe tumor (118 μ in largest diameter). The tumor consisted of large basophilic cells. These cells also gave an intense PAS reaction. M-A., ×150.

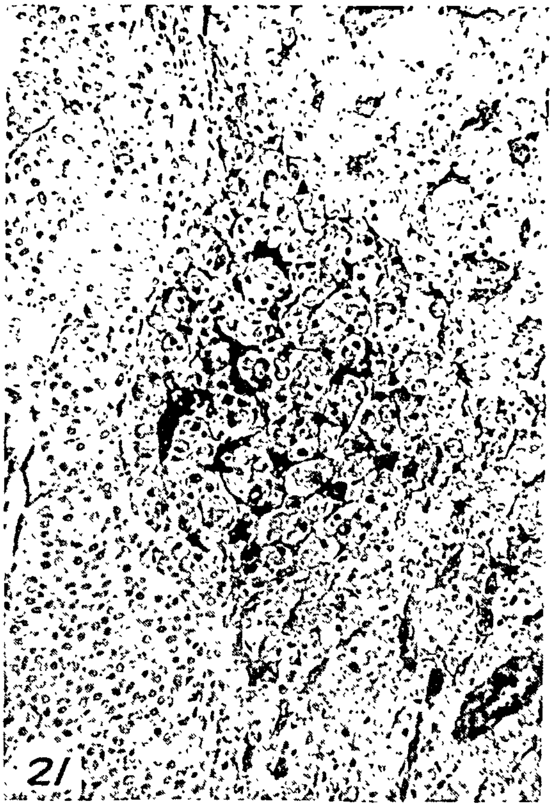
FIG. 17. Gigantic cell showing atypical nucleus from a pre-adenomatous area in the posterior lobe, 6 mo. following irradiation with 6300 rep. M.-A., ×500.

FIG. 18. Central area of chromophobic tumor in the anterior pituitary 22 mo. following 945 rep, showing enlarged nuclei with prominent nucleoli, small localized hemorrhage, and gigantic cell. Note the similarity of the atypical cells found in various parts of pituitary (compare with Figs. 12, 13, 15, 17, 18). M.-A., ×500.

FIG. 19. Small, indefinitely outlined tumor (160μ in diameter) which consisted of a cluster of brilliantly stained acidophils of varying size. Some of the acidophils were 3 times larger than normal. M.A., ×150.







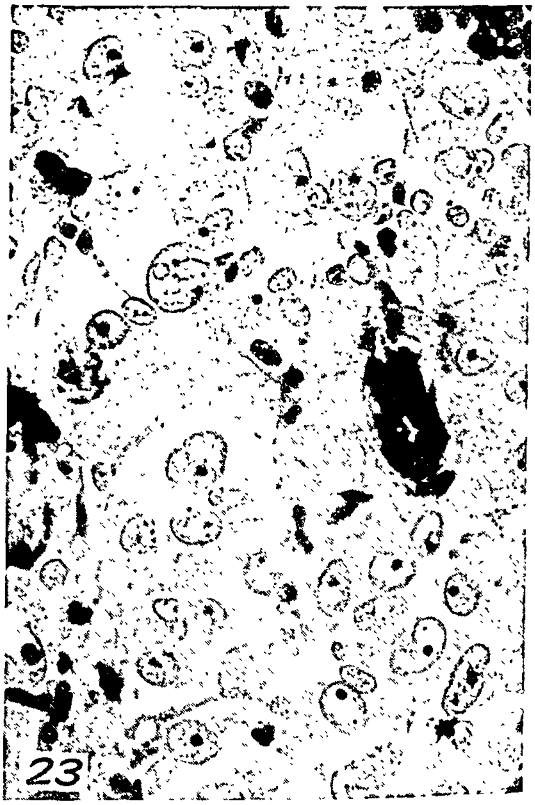


FIG. 20. Medium size tumor in the anterior pituitary 15 mo. following irradiation with 945 rep, consisting of heavily granulated and intensely stained acidophils. M.-A., ×500.

and 20). A few tumors were composed of both chromophobes and acidophils. Such areas of adenomatous change were often small and of low vascularity (Fig. 21).

In addition to such relatively circumscribed areas of adenomatous transformation in the anterior pituitaries of rats sacrificed between 12 and 24 months, clusters of parenchymal cells which appeared changed were observed in widely scattered areas. The cells in these clumps were sometimes enlarged, atypical in morphology and frequently in mitosis (Figs. 12 and 13). Such areas were considered pre-adenomatous.

Although most tumors occurred in the pars anterior, there were localized areas in the pars intermedia characterized by pronounced hypertrophy and altered morphology of the parenchymal cells (Figs. 14, 15 and 16). Both the nucleus and cytoplasm of individual cells were sometimes many times normal size. The changed cells in such areas were often arranged in cords, or formed acini with accumulation of fluid between the cells. Mitotic figures were found in such areas.

Tumor Incidence in Other Endocrine Organs

Multiple localized foci of adenomatous transformation were present in the adrenal cortex and in the thyroid of rats sacrificed during the second year (Table 2). Tumors were rare in the adrenal medulla, though a few pheochromocytomata were present both in controls and in groups receiving the low radiation to the pituitary gland. The incidence of adenomata of the parathyroid was low both in controls and in experimental groups. No adenomatous transformations were observed in the reproductive tract, with the exception of one testicular Leydig cell tumor in an irradiated group, and two small lesions resembling chorionic tissue in close relationship to the epididymis in a control group.

Incidence of Tumors in Non-Endocrine Organs

All rats maintained up to 26 months post-irradiation were examined carefully for presence of tumors in any part of the body, and any ab-

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Fig. 21. Typical, clearly outlined, small tumor (420 micra) in anterior pituitary, 22 mo. after irradiation with 945 rep. It consisted of basophils, quite uniform in appearance, showing strong PAS reaction. PAS, ×150.

Fig. 22. Central portion of a mixed-cell tumor (composed of chromophobes and acidophils) in the anterior pituitary, 23 mo. after irradiation with 945 rep. Note enlarged vesicular nuclei of chromophobes (C) and clusters of partially degranulated acidophils (A). M.-A., ×500.

Fig. 23. Central portion of a medium size basophilic tumor in the anterior pituitary, 23 mo. after irradiation with 945 rep. Note considerable variation in cell and nuclear size. In contrast with the basophils of the tumor depicted in the preceding figure, cells were irregular in size and some showed vacuolation of cytoplasm and nuclei. M.-A., ×500.

normalities detected were analyzed histologically (Table 4). The tumors were found chiefly between the ages of 12 and 24 months. Lymphosarcomas were the most common lesions, many of which appeared to originate in the lung. The incidence may have been somewhat lower in the irradiated groups. The few tumors of the pancreas observed were chiefly due to direct extension and invasion of lymphosarcomas arising in mesenteric lymph nodes (though one was probably of islet cell origin). There was no evidence of extension of lymphosarcomata through the blood channels. In one animal in which no localized lymphosarcomatous lesion was detected, lymphoid cells were present in large numbers in blood vessels of all organs examined and the interpretation of lymphoid leukemia was made. Isolated instances were observed in control and irradiated rats of adenomatous or fibro-adenomatous transformation in the pancreas, parotid and mammary gland.

TABLE 4. INCIDENCE OF TUMORS IN NON-ENDOCRINE ORGANS IN RATS SURVIVING MORE THAN ONE YEAR AFTER PITUITARY IRRADIATION

Type of tumors	Dosage rep	Total no. of rats	Tumors present	
			Number	Per cent
Lung lymphosarcomas	0	138	30	21
	945	39	3	8
	1575	42	2	5
	3150	65	7	10
	6300	19	1	5
Miscellaneous tumors (chiefly lymphosarcomas of mesenteric nodes)	0	138	11	8
	945	39	1	3
	1575	42	4	9
	3150	65	6	9
	6350	19	0	0

DISCUSSION

The incidence of tumors in pituitaries was greater in groups which had received the low doses of radiation than in controls, whereas at higher doses of radiation the appearance of tumors was virtually suppressed. Other abnormalities of the pituitary such as cysts and hemorrhages were also encountered more frequently after low radiation doses than in controls. The weights of the pituitaries of rats which received the low radiation doses increased slightly in the older groups, as can be seen in Table 1, though in preparing these averages pituitaries were eliminated which showed gross abnormalities. The increased incidence of pre-adenomatous and the multiple small areas of adenomatous change, together with the occurrence of small cysts and hemorrhages, offer a more probable explanation of the increase in weight of these pituitaries than does recovery or regeneration late after irradiation.

The number of tumors in other endocrine organs of the irradiated rats in no case exceeded those present in similar organs of controls of the same age; it is of interest to note, however, that no tumors were detected in pituitary-dependent endocrine glands, such as adrenal cortex and thyroid, which had become aplastic secondary to the destructive action of high deuteron irradiation of the pituitary. This is in keeping with the reduced incidence of tumors reported after surgical hypophysectomy (4).

The greater radiation sensitivity of the pituitary acidophils than of basophils described here was not noted in the earlier series, probably due to the subjection of the pituitary to higher radiation dosage which was followed by more prompt, serious damage to pituitary parenchyma (1, 5, 6, 7). This greater resistance of pituitary basophils was reflected in the status of endocrine function in the irradiated animals. Evidence for diminished hormone secretion from the anterior pituitary appeared in the dependent endocrine functions in the following sequence, as the dose or time interval increased: adrenal atrophy, with decreased cortical width, thyroid atrophy, slowed body growth and finally atrophy of the testes and dependent accessory sex organs. Sperm production was lost only under extremes of dosage and post-irradiation interval, when weight of the testis had been reduced to less than half normal. This persistence of testicular function can perhaps be attributed to the persistence of small amounts of pituitary tissue at high doses and the relatively great resistance of pituitary basophils even at high radiation dosages and over prolonged post-irradiation periods. The resistance of pituitary basophils to irradiation was evidenced to a lesser degree by the status of the thyroids.

Adiposity occurred during the second year post-irradiation after the lowest dose of radiation (945 rep). As noted previously this increase in weight in many rats of this group far exceeded the gradual weight gain in old normal rats. If it were assumed that the adopisity observed was due to radiation injury to the hypothalamus, it would have to be assumed that this was an effect manifesting itself only at the lowest doses of radiation and only after a prolonged period.

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EFFECT OF ETHIONINE ON THE CORTISONE-EVOKED STIMULATION OF TRYPTOPHAN PEROXIDASE-OXIDASE ACTIVITY[1]

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ABSTRACT

The activity of tryptophan peroxidase-oxidase was studied in rat liver following intraperitoneal injections of cortisone, ethionine, and methionine. Cortisone elicited the expected increase in activity, a dose of 50 mg. per kg. evoking a five-fold stimulation. Ethionine and methionine (0.77 mM.), on the other hand, did not alter the activity of this enzyme. Administration of ethionine with cortisone greatly reduced the stimulatory effect of the hormone. Coadministration of methionine with ethionine (both at a dosage of 0.77 mM.) restored the ability of cortisone to stimulate tryptophan peroxidase-oxidase activity. These results are taken to support the hypothesis that a *de novo* synthesis of this enzyme, believed to occur as a result of tryptophan administration, may also occur following the administration of cortisone.

THE activity of the enzyme tryptophan peroxidase-oxidase,[2] which catalyzes the conversion of L-tryptophan to L-formylkynurenine, has been shown to be increased in response to (a) administration of large doses of its substrate, tryptophan, and (b) administration of the adrenal corticoids, cortisone and hydrocortisone, or any agent which will elicit their secretion (1–4). Evidence has accumulated indicating that the stimulatory effect manifested by tryptophan is the result of an induced *de novo* synthesis of the enzyme (1, 5–9). Among the evidence in favor of this mammalian adaptation phenomenon is the demonstration by Lee and Williams (5) that ethionine inhibited the increase in TPO activity occurring as a result of tryptophan injection and that this inhibitory effect of ethionine could be at least partially reversed by the simultaneous administration of equimolar concentrations of methionine. Their results were interpreted to mean that ethionine suppresses the adaptive response of TPO to tryptophan through its well known inhibition of methionine incorporation into protein (10), thus interfering with the *de novo* synthesis of the enzyme.

Although the stimulation of TPO activity by adrenal corticoids has been observed in several laboratories (3, 8, 11), the mechanism of this effect remains to be elucidated. This investigation was undertaken to de-

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[2] Tryptophan peroxidase-oxidase is hereafter abbreviated as TPO.

termine the effect of ethionine on the corticoid-evoked stimulation in the belief that some insight into this hormonal response might be obtained through use of the ethionine-methionine interrelationship.

METHODS AND MATERIALS

Male Sprague-Dawley albino rats weighing approximately 200 grams were used. The rats were maintained on Purina laboratory chow fed *ad libitum;* however, only animals with matched food intakes were utilized in order to preclude possible effects due to variation in tryptophan consumption. Five hours prior to sacrifice, each animal was injected intraperitoneally with one of the following groups of materials: (1) 5 ml. of physiological saline, (2) L-methionine, 0.77 mM., (3) L-ethionine, 0.77 mM., (4) cortisone (saline suspension) 50 mg. per kg., (5) cortisone, 50 mg. per kg. and ethionine, 0.77 mM., (6) cortisone, 50 mg. per kg., ethionine, 0.77 mM. and methionine, 0.77 mM. Doses of ethionine and methionine used in this investigation conform to those of Lee and Williams (5) while the level of cortisone administered corresponds to the maximum amount given by Thomson and Mikuta during their dose-response studies (11).

Following decapitation, ten per cent liver homogenates of each animal were assayed for TPO activity by the method of Knox (12). Suitable aliquots of each homogenate were dialyzed against distilled water at 5° C for 36 hours prior to triplicate Kjeldahl nitrogen determinations by the method of Parnas and Wagner (13). In calculating nitrogen content, it was necessary to correct for volume changes which took place during dialysis of the homogenates. Specific activity of TPO is expressed as micromoles of kynurenine formed per hour per mg. of homogenate nitrogen.

The L-methionine, L-ethionine and cortisone used were obtained from the California Corporation for Biochemical Research.

RESULTS AND DISCUSSION

Rat liver TPO activities, five hours after injections of methionine, ethionine, cortisone, and combinations thereof, are presented in Table 1. Neither methionine ($P > 0.1$) nor ethionine ($P > 0.02$) significantly[3] altered the level of hepatic TPO activity from that found in the saline control animal. Cortisone administration, on the other hand, increased the activity of this enzyme five- to six-fold ($P < 0.001$), an increase which was somewhat greater than that obtained by other investigators (3, 11). The concomitant administration of 0.77 mM. of ethionine and cortisone resulted in a significantly lower ($P < 0.001$) TPO activity than that observed with cortisone alone. Injection of equimolar amounts of methionine and ethionine with cortisone restored the elevated TPO activity characteristic of rats treated only with cortisone ($P < 0.001$). Although the mean value was slightly higher, the enzyme activity of rats given cortisone, ethionine, and methionine did not differ significantly from the activity of the cortisone-treated animals ($P > 0.05$).

If, in the absence of evidence to the contrary, the usually accepted mode of ethionine inhibition is assumed to be valid under the conditions of this investigation, the following interpretation of the results obtained is possible. Cortisone may effect an increased TPO activity in the liver of the intact rat by means of a mechanism which involves stimulation of the *de novo* synthesis of the enzymatically active protein molecule from amino acid precursors available at the site of the "enzyme-forming system." The presence of ethionine, as administered, inhibits optimal incorporation of essential methionine into the enzyme protein and thereby partially suppresses the production of active TPO in response to cortisone. Methionine, if present in suitable quantities, acts to "reverse" the ethionine inhibition of TPO synthesis, presumably by competing with ethionine at the site of enzyme formation, thereby permitting the expected rise in TPO activity in response to the hormone. In the absence of cortisone, ethionine might not be expected to have a significant effect on the constititutive enzyme. Thus the results of this investigation lend support to the previously proposed (3) existence of a common step in the "hormone-induced" and "substrate-induced" mechanisms of TPO stimulation: viz. an increased *de novo* synthesis of the enzyme.

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[3] Accepting $P < 0.01$ as significant. Probability levels were calculated by means of the Student's "t" Test.

TABLE 1. RAT LIVER TRYPTOPHAN PEROXIDASE-OXIDASE ACTIVITY FOLLOWING ADMINISTRATION OF METHIONINE, ETHIONINE, AND CORTISONE

Group	No. of Rats	Materials Administered	TPO Activity* $\frac{\mu M.\ kynurenine}{Mg.\ N\ \ hour}$
I	10	Saline (controls)	0.0810 ± 0.0076
II	9	Methionine, 0.77 mM.	0.0991 ± 0.0077
III	9	Ethionine, 0.77 mM.	0.0528 ± 0.0087
IV	10	Cortisone, 50 mg./kg.	0.4415 ± 0.0386
V	9	Cortisone, 50 mg./kg. Ethionine, 0.77 mM.	0.2161 ± 0.0123
VI	10	Cortisone, 50 mg./kg. Ethionine, 0.77 mM. Methionine, 0.77 mM.	0.5532 ± 0.0509

* *Mean Values and Standard Error of the Mean.*

In certain *in vitro* studies utilizing rat liver slices (14) and "cell-free" preparations (3, 8), in which tryptophan stimulation of TPO activity was shown to occur, cortisone had no significant effect. Such evidence indicates that, although both the tryptophan and corticoid stimulations may involve *de novo* enzyme synthesis, the modes of action are probably independent. The mechanisms by which tryptophan and the corticoids initiate *de novo* TPO synthesis remain to be elucidated.

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EFFECT OF HORMONES UPON REGRESSION OF MUSCLE ATROPHY OF NERVOUS ORIGIN

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ABSTRACT

The effect of various hormones upon the regression of muscle atrophy of nervous origin has been studied in young rats. Unilateral nerve lesion was performed by crushing the obturator, sciatic and femoral nerves intrapelvically—a procedure which resulted in marked atrophy of the triceps surae muscle. Subsequent to complete functional recovery, treatment with hormones was initiated to see whether it would alter the course of the regression of the muscular atrophy.

STH, methyltestosterone, estradiol and thyroxin promote the rate of regression subsequent to reinnervation. Treatment with cortisol or 17-ethyl-19-nortestosterone, at the high dose levels used, exerts an opposite effect, while desoxycorticosterone has no influence.

From the results obtained in these experiments it can be concluded that, under certain experimental conditions, catabolic hormones, e.g., thyroxin and estradiol, can promote a circumscribed anabolic process, such as regression of muscle atrophy.

WHEN a skeletal muscle is deprived of its lower motor neuron, a process usually spoken of as denervation, a number of characteristic changes in the muscle take place (1). Such denervation is seen in many clinical conditions, most important of which are poliomyelitis and trauma to peripheral nerves. Unfortunately, both are common in normal times, but the incidence of the latter has been greatly increased in every war. Subsequent to nerve injury, under favorable conditions a functional motor reinnervation may take place when the progress of muscle atrophy is arrested and regression is initiated. But some degree of permanent atrophy is always to be anticipated after nerve injury. In spite of many new findings in this field (2–6) it is not possible, at the present time, to state precisely which factors are most important in determining the onset and rate of the development and regression of muscle atrophy. A disturbance of the trophic influence of the nervous system is generally assumed to exist (7), but the term "trophic influence" lacks physiological definition.

A review of the literature reveals many glowing reports of results obtained from treatment of post poliomyelitis, and similar conditions with local neural atrophies of the muscle tissue, by numerous and widely different forms of physical therapy (8). But it is important to point out that,

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based on the experimental results, considerable controversy exists as to the efficacy of electrical stimulation (9). Although it is possible to retard the development of muscle atrophy with induced activity, the onset is never completely prevented even though specific function is substituted by direct stimulation. Also, there is still the problem of finding a non-injurious agent which will be tolerated by the patient and will effectively retard atrophy and facilitate regeneration of muscle.

This report is concerned with experiments dealing with the effects of various hormones upon the regression of muscular atrophy of nervous origin. The experiments were carried out on the triceps surae muscle of the rat, because the extent and rate of development of muscle atrophy and of regeneration of nerves was found to run a remarkably constant course in this species (9, 10). One hind limb was paralyzed by unilateral crushing of the obturator, sciatic and femoral nerves and, thus, marked atrophy was produced. When functional recovery was complete, the degree of this neuromuscular atrophy was determined and the effect of hormones upon regression of the atrophy following reinnervation was studied.

EXPERIMENTAL MATERIAL AND TECHNIQUES

General experimental design. One hundred and ten young female Sprague-Dawley rats, with an average initial body weight of 51 gm. (range: 43–60 gm.), were subdivided into eleven equal groups. *Groups I and II* served as absolute controls. The rats of Group I were killed the day the experiment was started and were used as indicators of the normal initial values; those of Group II were killed on the last day (56th day) of the experiment to show the normal final values. *Groups III and IV* served as untreated controls in that they received no hormone treatment but were submitted to the unilateral nerve-crush procedure described below. The rats in Group III were killed on the 26th day of the experiment (when complete functional recovery was observed in all groups and hormone treatment was initiated). These animals served to show the average degree of atrophy that existed at this time. Group IV were killed at the end of the experiment to show the rate and degree of the spontaneous regression of muscle atrophy which takes place in the animals not treated with hormones. Rats in *Groups V–XI* were also submitted to the unilateral nerve crush procedure and treated as indicated in Table 2.

Throughout the period of observation, all the rats were fed exclusively on ground laboratory diet, Purina fox chow, and tap water. The food was ground, otherwise the animals would have had difficulty in eating it, as the teeth of all groups were clipped once every week—a procedure which prevented the animals from gnawing the denervated limb.

Production of muscular atrophy of nervous origin. In all rats of Groups III–XI, unilateral nerve lesion was performed under light ether anesthesia, through a suprapubic incision, on the first day of the experiment. The left obturator, sciatic and femoral nerves were intrapelvically crushed with narrow forceps, so that transparency of the nerve was produced at the site of crushing, which ensured the complete interruption of axons. In all instances, complete insensitivity and paralysis of the left limb resulted. This method of denervation leads to a most successful regeneration of the nerves (10, 11) and on the other hand, allows for the study of the regression of the muscle atrophy after reinnervation. The sensitivity and motility of the denervated limb were examined every second day after the surgical intervention. The recovery of sensory and motor function were separately assessed in the arbitrary scale of 0 to 3; the degeneration of the crushed nerves was estimated in an index of 0–6 (sensitivity plus motility). The index of 6 served as a criterion for "complete functional recovery," i.e., the sensitivity and the voluntary movement of the affected limb were both normal.

Treatment with hormones. On the 26th day of the experiment, treatment with various hormones was initiated in Groups V-XI as indicated in Table 2 and was continued until the termination of the experiment. The hormones were always injected in 0.2 ml. of water, subcutaneously.

Cortisol acetate was administered in the form of microcrystals of hydrocortisone acetate (Schering), at the dose of 1 mg./day (Group V).

Desoxycorticosterone acetate was administered in the form of Cortate (Schering), at the dose of 1 mg./day (Group VI).

STH (somatotrophic hormone) was administered in the form of the growth hormone (U.S. National Institutes of Health), in 2 daily injections of 1 mg. (Group VII).

Estradiol, Progynon (Schering), was administered in the form of micro-crystals, at the dose of 500 μg./day (Group VIII).

Methyltestosterone, Oreton-M (Schering), was given in the form of microcrystals, at the dose of 2 mg./day (Group IX).

17-Ethyl-19-nortestosterone was administered in the form of Nilevar (G.D. Searle & Co.), at the dose of 2 mg./day (Group X).

Thyroxin obtained from the British Drug Houses, was given at the dose of 250 μg./day (Group XI).

The evaluation of the experiment. On the 56th day of the experiment (i.e., 30th day after the initiation of hormone treatment) all rats were killed with chloroform and the sciatic nerve and the triceps surae muscle were dissected. After fixation in Susa solution, the muscles were weighed on an analytical balance. The means of these weights, the final body weights and the ratio of the muscle weight to body weight are given in Table 1 and 2 together with the standard errors. The differences between the contralateral muscles are especially indicated in a separate column, in which the weight of the muscle of the limb with nerve lesion is expressed as a percentage of the weight of the corresponding muscle of the intact limb. Following the weighings, the muscles were embedded in paraffin and stained with hematoxylin-phloxine for subsequent histological study. For the morphological studies of the sciatic nerve, Marchi stain was also used.

In all groups where as judged from the muscle weights a beneficial effect of the hormone treatment was observed (Groups VII, VIII, IX, XI), the findings were confirmed by measurement of muscle fibers. The diameter of 200 muscle fibers was measured for each triceps surae muscle (100 from transverse sections and 100 from longitudinal sections), histograms were drawn and the average diameter of the muscle fibers was determined. Fiber diameters were determined on both longitudinal and transverse sections, because even a small inclination of the cutting plane could considerably change the results obtained from transverse sections only, and a greater margin of error would result in the measurement of the smaller dimensions of the atrophic muscle fibers. The accuracy of the method was checked by measuring diameters (of both transverse and longitudinal sections) of the triceps surae muscles of the initial controls (Group I). The average value obtained from transverse sections of 100 muscle fibers was practically the same as that obtained by measurement of the longitudinal sections of 100 muscle fibers (left limb 17.9 μ:18.1 μ; right limb 18.0 μ:18.2 μ). Therefore, to obtain a high degree of accuracy, measurements from transverse *plus* longitudinal sections were taken throughout this work. The means of the average fiber-diameter values (with standard errors) and the percentile diameter differences of the contralateral muscles, are listed in Table 3.

RESULTS

The growth of intact muscle. The results summarized in Table 1 indicate that in early life (age: 4 to 12 weeks), the ratio of muscle weight to body weight increases in the rat; the triceps surae muscle gains weight faster than does the body as a whole.

Among the animals treated with anabolic hormones, only those treated with methyltestosterone showed muscle growth that paralleled that of somatic growth (cf. Table 2, Group IX). Administration of STH, while further facilitating muscle growth, produces a decrease in the ratio of muscle weight to body weight. 17-Ethyl-19-nortestosterone as well as desoxycorticosterone, at the high dose level used here, slightly diminished the normal muscle growth and somewhat decreased the muscle/body weight ratio. This decrease was more significant in the cortisol, estradiol and thyroxin treated groups, where the hormones, by their general catabolic effect, also produced a marked inhibition of the triceps surae growth.

The regression of muscle atrophy. In evaluating this data, we must keep in mind that at the time of the initiation of the treatment with hormones the average degree of muscle atrophy was 50.6%, as judged by the control animals (Group III). This figure had risen to 71.1% by the end of the experiment in the untreated rats (Group IV).

It can be seen from the results given in Table 2 (especially from the percentile differences of the contralateral muscles) that the effect of hormones on muscle which is in a process of regeneration is different from that on intact muscle. STH, methyltestosterone, estradiol and thyroxin promote the process of regression, while cortisol, and 17-ethyl-19-nortestosterone exert an opposite effect. Only desoxycorticosterone did not influence the rate of regression of the muscular atrophy subsequent to reinnervation. Nevertheless, it is important to point out that the subnormal ratio of the muscle weight to body weight due to the nerve lesion was only positively affected in the methyltestosterone and estradiol treated rats (compare the ratio of the muscle weight to body weight of Groups VIII and IX with Group IV). Especially in those treated with methyl-testosterone, this ratio was nearly normal (cf. Group IX with Group II).

The histologic observations can be summarized as follows: On initiation of the hormone treatment (on the 26th day after nerve crush) there was a marked atrophy of the affected triceps surae muscle, with the usual reduction of the gross bulk of the muscle and the dimensions of each of its constituent fibers. At this time, no degenerative process was observed. The microscopic examinations on the 56th day (30th day after complete functional recovery) also showed atrophy, but most of the fiber diameters had increased, while some other cells had even reduced their size and a few degenerated fibers with phagocytotic processes were observed. Therefore, there was greater variability of the fiber-diameters in the affected than in

TABLE 1. CONTROL VALUES FOR NORMAL SOMATIC GROWTH AND MUSCLE GROWTH (GROUPS I AND II) AS WELL AS FOR THE SPONTANEOUS RATE OF REGRESSION OF MUSCLE ATROPHY SUBSEQUENT TO REINNERVATION (GROUPS III AND IV)

Group	Treatment	Days of experiment	Body weight (gm.)	Triceps surae weight (mg.)			Triceps surae weight as percentage of body weight‡	
				Intact	Nerve lesion	%†	Intact	Nerve lesion
I	Control (initial)	0	51 ±1.4	254 ± 1.6	(255 ± 1.2)	100.3 ±0.44	49.8 ±1.42	(50.0 ±1.56)
II	Control (final)	56th	167 ±4.5	1123 ±15.9	(1119 ±16.4)	99.6 ±0.65	67.2 ±1.57	(67.0 ±1.35)
III*	Nerve lesion (initial)	26th	96 ±3.6	532 ±11.3	265 ± 9.1	50.6 ±1.15	55.4 ±1.19	27.6 ±0.92
IV	Nerve lesion (final)	56th	161 ±7.1	1112 ±23.2	791 ±15.1	71.1 ±1.10	69.0 ±1.75	49.1 ±1.22

* Group III was killed on the day that hormone treatment was started in Groups V–XI (cf. Table 2).

† The weight of the intact muscle is taken as 100 per cent.

‡ The muscle weights of the intact and nerve crushed sides are expressed as percentages of the final body weights

$$\left(\frac{\text{weight of muscle in mg.}}{\text{final body weight in gm.}} .100\right).$$

TABLE 2. EFFECT OF HORMONES UPON REGRESSION OF MUSCLE ATROPHY OF NERVOUS ORIGIN

Group	Treatment	Final body weight (gm.)	Triceps surae weight (mg.)			Triceps surae weight as percentage of body weight*	
			Intact	Nerve lesion	%*	Intact	Nerve lesion
V	Nerve lesion +COL-Ac	92 ± 6.1	605 ±19.8	374 ± 7.4	61.8 ±1.35	65.7 ±1.36	40.6 ±0.88
VI	Nerve lesion +DOC-Ac	166 ± 7.6	941 ± 9.4	668 ± 8.9	70.9 ±1.46	56.6 ±1.27	40.2 ±1.10
VII	Nerve lesion +STH	233 ±12.2	1342 ±11.0	1078 ±15.0	80.3 ±0.98	57.5 ±0.75	46.2 ±1.32
VIII	Nerve lesion +Estradiol	109 ± 5.5	688 ± 7.5	587 ± 5.2	85.4 ±1.06	63.1 ±1.58	53.8 ±1.09
IX	Nerve lesion +Methyltestosterone	165 ±14.8	1147 ± 7.9	1031 ±12.1	89.8 ±1.25	69.5 ±1.15	62.4 ±1.43
X	Nerve lesion +17-Ethyl-19-Nortestosterone	162 ±10.9	1014 ±15.2	689 ±12.7	67.9 ±0.79	62.5 ±1.40	42.5 ±1.26
XI	Nerve lesion +Thyroxin	131 ± 8.4	696 ±18.1	557 ±13.5	80.0 ±1.21	53.1 ±0.88	42.5 ±1.19

* For further interpretation, see the descriptions of the corresponding columns in Table 1.

the corresponding intact muscle. The same is true also for all the hormone treated groups, which did not show any other marked histologic changes. In regard to nerve studies, at the end of the experiment no areas of degeneration in the sciatic nerve, distal from the site of crushing, were observed.

From the figures in Table 3, it can be stated that the results obtained with the muscle weight studies were, in general, confirmed by fiber-size measurements in the triceps surae muscle. The regeneration-facilitating effect of STH, however, seemed to be more evident and, on the other hand, the positive influence of thyroxin proved to be less significant, as judged by determinations of the average diameters in muscle fibers.

TABLE 3. RESULTS OF THE STUDIES ON MUSCLE-FIBER DIAMETERS

Group	Treatment	Fiber size in triceps surae (μ)		
		Intact	Nerve lesion	%*
I	Control (initial)	18.1 ±0.47	(18.0 ±0.55)	99.4
IV	Nerve lesion (final)	30.7 ±0.61	20.8 ±1.56	67.7
VII	Nerve lesion +STH	34.9 ±0.55	29.7 ±1.41	85.3
VIII	Nerve lesion +Estradiol	24.6 ±0.42	20.2 ±1.21	82.1
IX	Nerve lesion +Methyltestosterone	31.6 ±0.56	27.3 ±1.12	86.3
XI	Nerve lesion +Thyroxin	26.1 ±0.36	19.9 ±1.38	76.2

* The average fiber-diameter of the intact muscle was taken as 100%.

DISCUSSION

It is evident from the summary of our findings that among the hormones used, methyltestosterone exerted the most beneficial effects upon the regression of muscle atrophy of nervous origin. Since 17-ethyl-19-nortestosterone proved to be a highly active anabolic steroid in man (12) and its anticatabolic properties have also been experimentally demonstrated (13), we decided to compare its effect on muscular tissue with that of the relatively more virilizing methyltestosterone. Our results now indicate that at the high dose level used none of these hormones stimulate the somatic growth and their effect is different upon the intact and especially on the regenerating muscular tissue: methyltestosterone facilitates the regression of muscle atrophy to near completion, while 17-ethyl-19-nortestosterone produces a marked inhibition of this process. It seems possible that the anabolic effects of testoid compounds upon the muscular tissue depend not only upon the dose and duration of the treatment (14,15: for further literature see Ref. 16), but also on other, as yet uncertain, conditions.

The regeneration-facilitating effect of STH is also satisfactory, because based on a comparison with other observations (17, 18) it can be assumed that the decrease of the ratio of the muscle weight to body weight produced by STH in the present investigation is characteristic only during an early period of life and is, thus, conditional.

It is important to point out furthermore that estradiol, despite its general catabolic properties, was unexpectedly found to be effective in facilitating the regression of muscle atrophy. Our experiment clearly demonstrates the enhanced regression after estradiol treatment, but as only intact female rats were used, there is no indication of the mechanism involved.

As regards the effect of thyroxin, it is worthy of mention that previous experiments demonstrated that this hormone accelerates the rate of atrophy of denervated muscle (6, 9). It is believed that thyroxin administration exerts this effect by acting as an accelerator to the catabolic processes involved in the disintegration of the cytoplasm of the denervated muscle. Therefore, the observation that thyroxin does not exert the same unbeneficial effect on the regenerating muscle is worthy of attention.

The finding that cortisol, by diminishing the normal regression process, resulted in a more marked degree of muscle atrophy between all groups may be compared to recent findings. These demonstrated not only the aggravation of the development of muscle atrophies of nervous and disuse origins (3, 6), but also the production of a circumscribed muscular atrophy (2, 4) after cortisol treatment.

In considering the present findings, it should finally be noted that by employing a nerve crush method similar to that used in these studies, we were able to demonstrate that some degree of permanent atrophy is to be anticipated after nerve injury. An experiment was performed on 8 rats with an initial body weight of 53 ±3.4 gm. Five months after the nerve lesions were performed, the following results were observed: Final body weight, 246 ±12.6 gm.; intact triceps surae weight, 1608 ±10.6 mg.; contralateral muscle weight (on the side with nerve lesion), 1151 ±14.2 mg.; degree of muscle atrophy (percentage difference), 71.5%; ratio of the intact muscle weight to body weight, 65.4%; ratio of affected muscle weight to body weight, 46.8%.

Comparison of these findings with those after STH, methyltestosterone, estradiol and thyroxin treatments shows that over a considerably short period these hormones are able to increase the regression of the muscle atrophy to above the final degree which is seen in animals not treated with hormones.

It is also of interest to note that the data obtained in these experiments allow for the conclusion that, under certain conditions, catabolic hormones (e.g., thyroxin and estradiol) can even promote a circumscribed anabolic process, such as the regression of atrophied muscle tissue.

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EFFECT OF PROGESTERONE AND ESTRONE ON THE INCIDENCE OF CONGENITAL MALFORMATIONS DUE TO MATERNAL VITAMIN E DEFICIENCY[1]

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ABSTRACT

In a series of experiments 299 Holtzman rats were used to determine the effect of progesterone and estrone on the incidence of congenital abnormalities induced by marginal maternal vitamin E deficiency. It was observed that daily subcutaneous injection of 4 mg. of progesterone or 2 μg. of estrone, singly or in combination, from the third day of gestation till term, in addition to 2 mg. of *d,l*-alpha-tocopherol acetate supplementation on the 10th day, resulted in a reduction of the weight gain of the mother rats during gestation, the average number of implantation sites and the percentages of normal and abnormal young, with concomitant increase in the incidence of resorption. This abolition of the appearance of congenital abnormalities was further substantiated unequivocally when a single dose of 4 mg. of progesterone or 2 μg. of estrone was given by gavage together with 4 mg. of vitamin E on the 10th day of gestation. Similar injections of 1 mg. of progesterone and/or 0.5 μg. of estrone as well as the injections of 4 mg. progesterone and 10 mg. estrone did not produce any significant effect. Thus, the administration of progesterone or estrone at certain levels continuously or by a single dose either reduced or abolished the incidence of congenital malformations in maternal avitaminosis E.

PREVIOUS studies have shown that congenital malformations can be produced in the rat by maternal vitamin E deficiency (1, 2). Some preliminary observations on the histological and other changes in the term fetuses have been described (3, 4). Gross observations of the abnormal embryos from the 11th day of gestation onwards have recently been reported (5). The literature on the interrelationship between vitamin E and hormones was reviewed by Mason (6), who concluded that the preponderance of experimental evidence up to 1944 exonerated the anterior pituitary and thyroid as instigators of reproductive and other disturbances in vitamin E deficiency. However, recently Kaunitz (7) showed by ovarian transplantation experiments that there was some unspecific ovarian dysfunction in prolonged vitamin E deficiency. This fact was also borne

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out by the finding that the gonadotrophin content of the anterior pituitary of old, vitamin E-deficient male and female rats was significantly increased (8). Furthermore, after studying the effects of injection of alpha-estradiol, progesterone and tocopherol singly or in combination upon the uterine pigment formation of spayed, young vitamin E-deficient rats Kaunitz and associates (9, 10) suggested that progesterone and the tocopherols seemed to have functionally similar effects in preventing pigment formation in estradiol-injected, spayed females. Since the interrelationship between vitamin E and hormones is still in a state of controversy, it was deemed interesting to determine the effect of estrone and progesterone on the incidence of congenital abnormalities induced by maternal vitamin E deficiency.

EXPERIMENTAL METHODS

A total of 299 rats were used in the experiments reported here. The procedure used in the production of congenital abnormalities in maternal vitamin E deficiency has been reported previously (2, 5). Briefly, weanling female rats of Holtzman strain weighing approximately 40 gm. were put on a vitamin E-deficient ration consisting of 49% dextrin, 22% lard, 18% vitamin-free casein, 5% brewers' yeast, 4% salt mixture (U. S. No. XIV), and 2% cod liver oil. When they attained a weight of approximately 175 gm., they were mated with males which were fed stock pellets. After mating, as evidenced by the presence of sperm in the vaginal smear, they were assigned to different groups. Positive evidence of implantation was ascertained by observing the presence of erythrocytes in the vaginal smears during the second week of gestation. Two mg. of *d,l*-alpha-tocopherol acetate in 1 ml. of corn oil (Mazola) was given to each of the rats by stomach tube on the 10th day of gestation. This procedure has been used successfully in the production of abnormal young from vitamin E-deficient mothers. The rats with the supplementation of vitamin E only constituted the uninjected group. Seven groups of rats were injected daily subcutaneously with different levels of progesterone and estrone in 0.2 or 0.5 ml. of corn oil separately or in combination from the third day of gestation till term in addition to the vitamin E supplementation. Two groups of rats were injected daily with either 0.2 or 0.5 ml. of corn oil, the vehicle for the hormones, for the same duration to serve as controls. Also, positive controls which received 2 mg. of *d,l*-alpha-tocopherol acetate daily during the first 5 days of gestation, and negative controls which did not receive any vitamin E supplementation were used. All rats were laparotomized on the 21st day of gestation. The endocrine organs of many of these rats were weighed and fixed for histological examination, the results of which will be reported later.

For analysis of the statistical significance of the incidence of abnormalities and others the chi-square test was used. The significance of difference between groups with regard to numbers of implantation sites was assessed by means of the "Student" t test (11).

RESULTS

The results of the first series of experiments are presented in Table 1. The rats that had received hormone injections gained less weight during gestation than those without the injections, with those receiving 4 mg. of progesterone and 10 mg. of estrone gaining the least weight. Therefore, it appears that the administration of progesterone and estrone under the conditions of the present experiments did not improve the general health of the pregnant rats.

The percentage of normal fetuses in the uninjected group was reduced to 16.7% from the positive control of 90.3%. Further reduction of the percentage of normal fetuses occurred in rats receiving either 1 or 4 mg. of progesterone or 2 μg. of estrone. There were no normal live fetuses in rats receiving 0.5 μg. of estrone or various combinations of estrone and progesterone. The percentage of normal fetuses in the corn oil groups was about the same as that in the uninjected group. The negative control group receiving no tocopherol supplement yielded, as usual, no normal young.

TABLE 1. EFFECT OF PROGESTERONE AND ESTRONE ON THE INCIDENCE OF CONGENITAL ABNORMALITIES IN VITAMIN E-DEFICIENT RATS THAT HAD RECEIVED 2 MG. D,L-ALPHA TOCOPHEROL ACETATE ON THE 10TH DAY OF GESTATION

Treatment	Rats bred No.	Wt. gain during gestation, gm.	Live fetuses		Dead fetuses		Implantation sites[1]	
			Normal %[6]	Abnormal %[6]	Dead %[6]	Resorbed %[6]	Total No.	Average No.
Positive control[2]	46	73	90.3	0	0	9.7	420	9.1±0.9[8]
Uninjected	37	45[3]	16.7	5.3	1.0	77.0	280	7.8±2.4
Progesterone 1 mg.	14	28	2.5	0.8	0	96.7	119	8.5±2.7
Progesterone 4 mg.	19	35	9.3	0.9[7]	0.8	89.0	129	6.8±2.5[7]
Estrone 0.5 μg.	16	17	0	0	0	100.0	20	6.7[4]±2.0
Estrone 2 μg.	11	32	10.0	0[7]	0	90.0	68	6.1±2.2[7]
Progesterone 1 mg. +estrone 0.5 μg.	8	23	0	0	0	100.0	50	6.3±2.8
Progesterone 4 mg. +estrone 2 μg.	14	22	0	0[7]	0	100.0	76	5.4±2.5[8]
Progesterone 4 mg. +estrone 10 mg.	13	15	0	0	0	100.0	—	—
Corn oil 0.2 ml.	13	35	17.0	0[7]	1.0	82.0	82	6.3±2.4[7]
Corn oil 0.5 ml.	22	37	16.0	0[8]	1.0	83.0	179	8.0±2.8
Negative control[5]	40	26	0	0	0	100.0	359	9.0±1.9[7]

[1] Including those of live and non-resorbed dead fetuses.

[2] The rats in this group received 2 mg. daily of d,l-alpha-tocopherol acetate during the first 5 days of gestation.

[3] Average of 33 rats.

[4] Average of 3 litters.

[5] Vitamin E-deficient group. The rats did not receive any vitamin E supplementation.

[6] Calculated on the basis of total number of implantation sites, instead of on the number of live young obtained.

[7] $P < .05$ when compared with the uninjected group.

[8] $P < .01$ when compared with the uninjected group.

The most interesting result was a marked reduction in or abolition of the incidence of congenital abnormalities as one of the consequences of the hormone treatment. Calculated on the basis of total number of implantation sites, the incidence was 5.3% in the group without hormone treatment, but it was 0.8% and 0.9% in the groups with 1 and 4 mg. of daily progesterone injection, respectively. Also, no abnormal fetuses occurred in the groups given estrone treatment, singly or in combination with progesterone. The differences between the uninjected group and the group receiving 4 mg. progesterone or 2 μg. estrone, separately or in combination, were significant at the 5% level. Those between the uninjected and the corn oil control groups were also significant at the 1% and 5% levels, respectively.

The percentages of resportion were all increased in the groups injected either with the hormones or corn oil alone, with the groups receiving 0.5 μg. estrone and various combinations of progesterone and estrone showing complete resorption. The average number of implantation sites for the uninjected group was 7.8, which was significantly lower than in either the positive or negative control groups. However, the average number of implantation sites, as well as the percentage of abnormal young, was significantly reduced when 4 mg. progesterone or 2 μg. estrone was injected singly or in combination during gestation.

Inasmuch as the continuous injection of either 0.2 ml. or 0.5 ml. of corn oil alone to the rats from the third day of gestation till term also abolished the appearance of abnormal young, the efficacy of the hormones in this respect might appear to be questionable. In order to pinpoint the effect of the hormones and to obviate the complication of the effect of continued injection of the vehicle, corn oil, another series of experiments was undertaken in which the rats were given by gavage a single dose of 4 mg. of progesterone or 2 μg. of estrone together with 4 mg. alpha-tocopherol acetate dissolved in 1 ml. of corn oil on the tenth day of gestation. The use of 4 mg. of tocopherol was chosen in this series of experiments instead of the 2 mg. level since a higher incidence of abnormalities had been obtained with the 4 mg. level of supplementation (3).

TABLE 2. EFFECT OF A SINGLE DOSE OF PROGESTERONE OR ESTRONE ON THE INCIDENCE OF CONGENITAL ABNORMALITIES IN THE OFFSPRING OF VITAMIN E-DEFICIENT RATS

Treatment on 10th day of gestation	Rats bred No.	Wt. gain during gestation, gm.	Live fetuses		Dead fetuses		Implantation sites[1]	
			Normal %[1]	Abnormal %[1]	Dead %[1]	Resorbed %[1]	Total No	Average No.
Vitamin E 4 mg	20	60	17.7	12.8	0.4	69.1	226	11.3 ± 5.2
Vitamin E 4 mg. + progesterone 4 mg.	11	43	7.5[2]	0[2]	0	92.5[2]	80	8.9[3] ± 3.3
Vitamin E 4 mg + estrone 2 μg.	15	56	26.2[2]	0[2]	0.8	73.0	130	8.7 ± 2.1

[1] Calculated on the basis of total number of implantation sites
[2] P <.01 when compared to groups with 2 or 4 mg vitamin E alone.
[3] Average of 9 litters

The results are presented in Table 2. With a single oral dose of 4 mg. progesterone or 2 μg. estrone in conjunction with 4 mg. alpha-tocopherol acetate on the 10th day of gestation there was a complete abolition of the occurrence of congenital malformations in the live young produced while those rats receiving 4 mg. of tocopherol alone produced 12.8% abnormal fetuses. In the case of progesterone treatment there was a significant reduction in the percentage of the normal young with a concurrent increase in the percentage of resorption similar to those obtained in the first series of experiments. However, in the estrone experiment there was an increase in the percentage of normal young while the incidence of resorption remained about the same as in the control groups. There was no significant difference in the average number of implantation sites, nor in the maternal gain in weight during gestation, between the experimental and control groups.

DISCUSSION

As far as the percentage of abnormal young is concerned, it is apparent from the data in Table 1 that the injection of corn oil or ovarian hormones in corn oil from the 3rd day of gestation until term reduced the incidence of congenital abnormalities induced by feeding mother rats a vitamin E-deficient ration. It is not certain, from the data of this experiment, whether the reduction of abnormal fetuses was due to the injection of corn oil or the hormones *per se*. It should be pointed out that in the case of corn oil injections, the abolition of abnormal fetuses occurred with corresponding increase in the percentage of resorption without affecting the percentage of normal young. The effects of corn oil in this respect could be due to either one of the following possibilities. Firstly, a change of the critical balance of the borderline deficiency of vitamin E, which is necessary for the production of congenital abnormalities, could have occurred. The presence of a trace amount of vitamin E (12) in corn oil might have changed the vitamin E content of the blood of the mother to such an extent that the delicate balance by which abnormality could occur was shifted slightly. Secondly, it is possible that the corn oil might contain an unknown factor which could prevent the occurrence of abnormal fetuses when used in quantities larger than 1 ml. Further investigation is required to elucidate this point.

The reduction or abolition of abnormalities in live fetuses by the injection of ovarian hormones in corn oil was not of the same nature as that brought about by corn oil alone as shown by the fact that in the former the percentage of resorption was greatly increased with slight to drastic reduction of the percentage of normal young. In the case of the combined injection of 0.5 μg. of estrone and 1 mg. of progesterone, or 2 μg. of estrone and 4 mg. of progesterone, or 10 mg. of estrone and 4 mg. of progesterone, complete resorption occurred, similar to the reproductive performance of female rats in the negative control group receiving no supplemental tocopherol. The physiological significance of these results are not entirely clear. It would appear that the continued injection of these hormones could inactivate the minute amount of tocopherol in the circulatory system resulting in total vitamin E deficiency.

Results from the second series of experiments where the hormones were administered by gavage at a level of 4 mg. of progesterone or 2 μg. of estrone together with 4 mg. of tocopherol in corn oil in a single dose on the 10th day of gestation offer more clear-cut effects. The use of 4 mg. of tocopherol was justified inasmuch as this dose tends to increase the incidence of abnormalities. It is quite apparent from data presented in Table 2 that the oral administration of either 4 mg. of progesterone or 2 μg. of estrone abolished the occurrence of the abnormal young. While both of these hormones abolished abnormalities, the mechanism of the abolition was probably of a different nature in each case. In the case of progesterone administration there was an increase in resorbed fetuses. Ershoff (13) noted that the daily injection of 150 I.U. of estrone, 4 mg. of progesterone or 2 mg. of lactogenic hormone to vitamin E-deficient rats from the 6th to the 16th day of pregnancy resulted in total resorption of fetal sites by the time of autopsy on the 16th day. As far as increasing the incidence of resorption is concerned it seems that the results obtained here are in accord with those of Ershoff although a physiological explanation is still not readily available.

In the case of estrone administration, the percentage of resorption was maintained at the level of the tocopherol treated group but the percentage of normal young was increased with total abolition of abnormal young. Thus, the oral administration of a single dose of 2 μg. of estrone on the 10th day appears to be effective in counteracting the teratogenic effect brought about by marginal vitamin E deficiency. It is quite possible that the teratogenic effect of this type of marginal nutritional deficiency was partially induced by an inadequate secretion of estrogens. Evidence on this point has been forwarded by Nelson, Lyon and Evans (14) who showed that estrogenic hormones afforded marked protection during pregnancy in pyridoxine-deficient rats.

Finally it might be interesting to point out the difference in the average number of implantation sites of the groups following the hormone administration between the first and second series of experiments. In the first series where the subcutaneous injection of the hormones was started on the third day post coitus, i.e., before implantation took place, there was a significant reduction in the average number of implantation sites in the groups receiving 4 mg. progesterone or 2 μg. estrone, separately or in combination. In the second series when the hormone administration took place on the 10th day when implantation had occurred, there was no significant reduction in the average number of implantation sites. Our results from estrogen experiments confirm the previous observations of Courrier and Gros (15) in the cat. These authors found that if estrogen is given in the interval between insemination and implantation it prevents the implantation and neutralizes the progestational changes in the uterus. However, after implantation, gestation is very difficult to interrupt with estrogen.

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COMPARATIVE EFFECTIVENESS OF ESTROGENS ON FLUID IMBIBITION AND GROWTH OF THE RAT'S UTERUS[1]

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ABSTRACT

Five natural estrogens (estradiol-17B, estrone, estriol, equilin and equilenin) and one synthetic estrogen (diethylstilbestrol) were compared as to effectiveness in promoting fluid imbibition and growth of the uterus in 22-day old rats. Graded amounts of each estrogen were injected subcutaneously as a single dose in 0.1 cc. sesame oil and uterine responses observed at intervals up to 72 hours. Both quantitative and qualitative differences were found with respect to dose required for a response and speed, duration, and extent of the reaction. Some estrogens are more effective for fluid imbibition and others more active for growth and superiority for one does not imply superiority for the other.

It was found that the minimal dose in micrograms required to produce 33⅓% increase in uterine wet weight at six hours (Astwood unit) is for estradiol, 0.025; estriol, 0.029; diethylstilbestrol, 0.078; equilin, 0.312; estrone, 0.45; and equilenin, 0.546. The dosage required to produce 70% gain in uterine wet weight at six hours is estriol, 0.078; estradiol, 0.1; diethylstilbestrol, 0.156; equilenin, 0.625; estrone and equilin, 1.25. The dose at which maximal imbibition occurs at four hours rather than at six hours is estriol, 0.039; estradiol, 0.15; diethylstilbestrol, 0.625; equilin, 5; and equilenin and estrone, 50.0. The order of potency for uterine growth between the thirtieth and fortieth hours by the dose required for maximal fluid imbibition on the sixth hour was found to be diethylstilbestrol, 0.1; estradiol, 0.1; equilin, 1.25; estriol, 5.0; estrone, 1.25; and equilenin, 1.25. While the order of effectiveness for growth at large doses, up to 50 μg., was diethylstilbestrol, estradiol, equilin, estrone, equilenin and estriol.

ESTROGENIC compounds, both natural and synthetic, vary widely in their capacity to promote growth of the different structures of the female reproductive tract. There is also a marked species difference in responsiveness. Of two animals most commonly used, the mouse is much more sensitive than the rat. However, comparisons of activity are usually based upon a single reaction in a particular test animal, and the response most frequently selected in the past has been vaginal cornification. The chief advantage of this method is its simplicity and the fact that the same animals can be used repeatedly, but the data obtained are in large measure qualitative and subjective in that they depend upon the evaluation of cellular contents of a vaginal lavage. There is also a disadvantage in that

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it is difficult to excise tissues accurately from the vaginal wall for biochemical studies. A structure best suited for comparative studies of the physiology of estrogens should be one that is not only responsive, but also discrete and easily accessible. The uterus comes most nearly meeting these requirements.

Astwood (1) studied the sequence of changes in fluid content and weight of the uteri of 21 to 23-day old rats following single injections of graded doses of estradiol. This procedure not only made it possible to evaluate the response in terms of dosage but also related the succession of events with respect to time. He found that the response to a given dose of estradiol followed a characteristic pattern. The first noticeable effect was hyperaemia associated with accumulation of fluid in the tissues and uterine lumen. This phase of the response was maximal at six hours, followed by a decrease in weight due to a partial resorption of fluid between the ninth and twelfth hours. At this point the uterus entered into a growth phase which attained a maximal extent by the thirtieth hour.

The time-relationship of uterine responses to estrogen has been used by others in more recent research. Notable among these are the observations by Telfer (2) on the influence of estradiol on nucleic acids, respiratory enzymes and the distribution of nitrogen in the rat uterus, and the experiments by Mueller (3) on the incorporation of radio-active amino acids in uterine tissue under the influence of estrogen. This problem has been approached in the present study by comparing the individual capacities of five natural estrogens (estradiol-17B, estrone, equilin, and equilenin) and one synthetic compound (diethylstilbestrol) to induce fluid imbibition and growth of the uterus.

METHODS

The animals used in the present investigation were normal sexually immature 22-day old female rats of the Harvard strain weighing 40 to 50 grams. The estrogens were furnished in crystalline form by courtesy of the manufacturer,[2] and a stock solution of each was prepared by serial dilution. The estrogens at all dosage levels were administered subcutaneously in 0.1 cc. of sesame oil and the animals were killed at selected times following a single injection.

The animals were killed by cervical dislocation, the uteri were immediately dissected out and freed from the adhering tissue, weighed on a torsion balance and placed in a tared vessel for dry weight determinations. For dry weights, the tissues were placed in an oven at a temperature of 100 to 115 degrees centigrade and weighed twenty-four hours later on a microbalance. Nitrogen determinations were made at the beginning of the study but these values so closely paralleled absolute dry weights that this was not included as a routine procedure.

In those instances where expressed uterine weights were desired, the uteri were removed as above, weighed, and then nicked with scissors, the luminal contents gently pressed out on moist paper toweling and reweighed. All uterine weights, unless otherwise stated, are for the tissue plus the luminal contents. The wet weights of uteri that were distended with luminal fluid, are slightly less than true values, as in the process of removal it is impossible to prevent the loss of a small portion of the fluid when the uterine cervix is cut.

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[2] We are very grateful to Park, Davis and Company, Schering Corporation and Ayerst Laboratories for generous gifts of natural estrogens.

A four-hour and six-hour dose response curve was determined for each estrogen for the study of fluid imbibition. Two dosage levels were used in evaluating the relative growth promoting properties of the different estrogens. A high dosage of fifty micrograms and a low dosage, determined by using the minimal amount of hormone giving a maximal fluid imbibition within six hours.

Approximately eight to ten animals were used to establish the position of each point on a particular curve. The data on uterine wet and dry weights and percentage of water were analyzed statistically to determine the degree of uniformity within each group by using the formula derived for use with calculating machines (8).

$$\text{Standard error} = \sqrt{\frac{\dfrac{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}}{n-1}}{n}}\,.$$

RESULTS

Fluid imbibition

Fluid imbibition increases proportionally with the dose of estrogen administered and the following is noted when a graded series of doses is given and the uteri weighed six hours later: a) The percentage differences in weight gained are greatest at low dosages, and decrease as the tissue reaches its maximal ability to respond, b) Amounts of an estrogen greater than that required for a maximal response at six hours not only do not produce a further gain in weight but may reach a point at which the response is less than that for a smaller dose (Figs. 1–6).

The drop in uterine weight occurring on the sixth hour at high dosages of estrogens would appear to be due to a self-inhibitory effect, but such is not the case. Uterine weights obtained at the end of four hours for high dosages may be higher than those at six hours for the same dose (Figs. 1–6). Also, when the four-hour responses are examined it is again noted that in many instances, as in the six-hour test, there is a falling off of uterine weights with higher dosages. The same explanation may again be applied, as in certain instances three-hour responses were found to be higher than those for the corresponding four- or six-hour test (Fig. 11). Thus, it can be stated: as the dosage of an estrogen is increased the time of maximal fluid imbibition occurs earlier than that obtained with smaller dosages.

All gains in weight during the first six hours are due mainly to the accumulation of fluid in the lumen of the uterus. Dry weights for these uteri show an increase over those of the control but this is misleading. The gain is due to the solid material present in luminal fluid, as shown by the fact that when luminal fluid is expressed the uterine dry weight is essentially the same as that for the untreated control.

The percentage of total water parallels uterine wet weight. As uterine

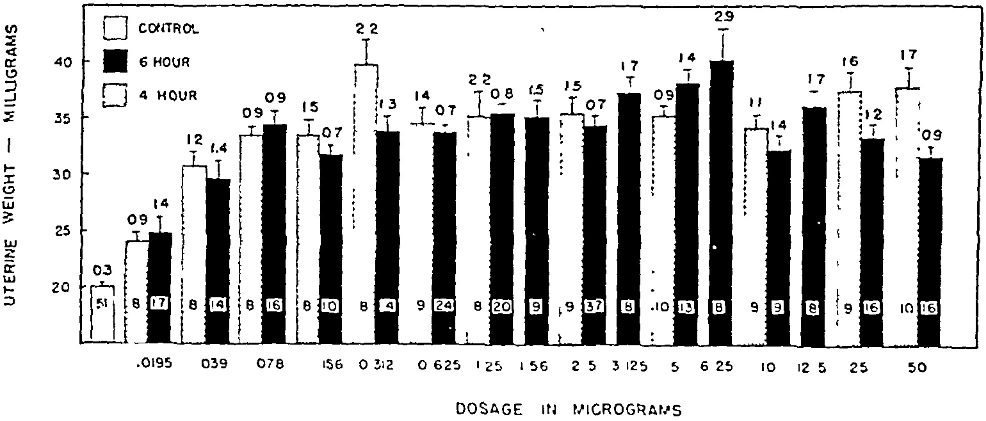

FIG. 1. Four and six hour response to estriol. The standard errors are shown at the top of the bars in figures 1 to 6, and the number of animals used is indicated on the base of each bar.

weight increases with successive dosages so does the percentage of water. Likewise, when the amount of water is decreasing, during the period of resorption, uterine wet weights also decrease.

Dry weights show a different stituation when the six and nine hour responses to a low dosage are compared with their percentages of water. The percentage of water differs by three percent in the total amounts while the absolute dry-weights are the same. A possible explanation is, that solid material is carried into the lumen as a part of the luminal fluid, but during resorption mostly water is removed, resulting in a fluid of greater concentration.

Estrogens differ in their ability to promote fluid imbibition. To facilitate comparisons, estradiol was used as the standard. An Astwood unit is that

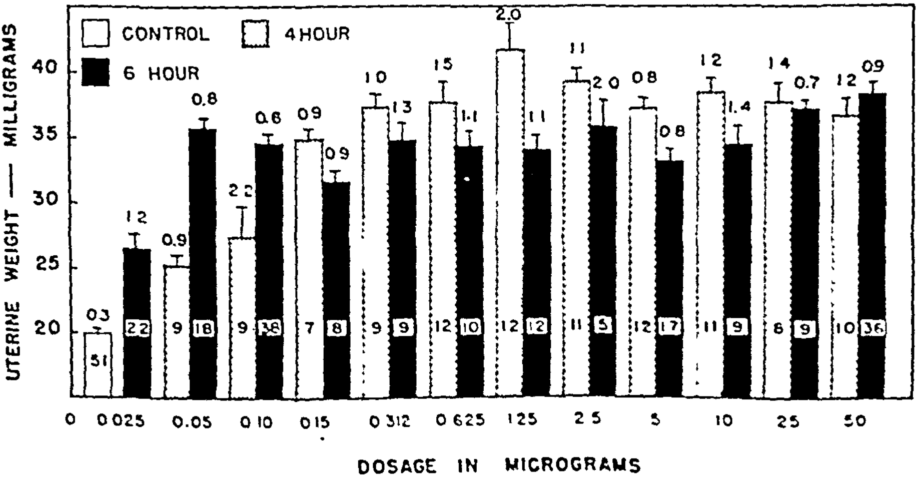

FIG. 2. Four and six hour response to estradiol.

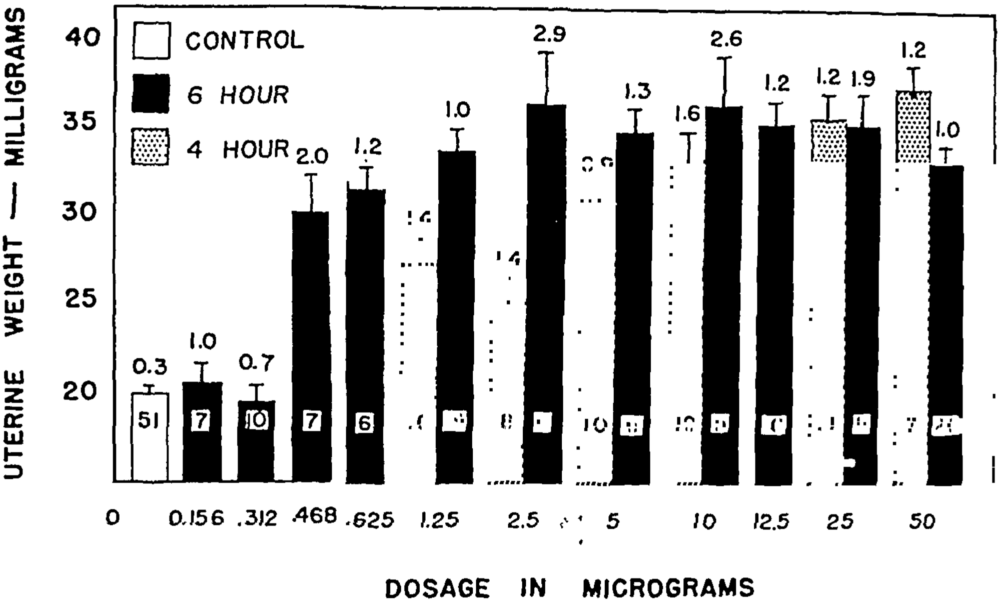

FIG. 3. Four and six hour response to estrone.

amount of estrogen which will elicit an increase in uterine wet weight of 33⅓% in six hours. This unit is equal to 0.025 μg. of estradiol. The order of estrogenic potency from strongest to weakest, in micrograms equivalent to one Astwood unit, is as follows: estradiol, 0.025; estriol, 0.029; diethylstilbestrol, 0.078; equilin, 0.312; estrone, 0.45; and equilenin, 0.546. Estradiol and estriol are almost equal and they in turn are about three times as strong as diethylstilbestrol, 12½ times stronger than equilin, 18 times as strong as estrone, and 21.8 times stronger than equilenin in producing uterine fluid imbibition.

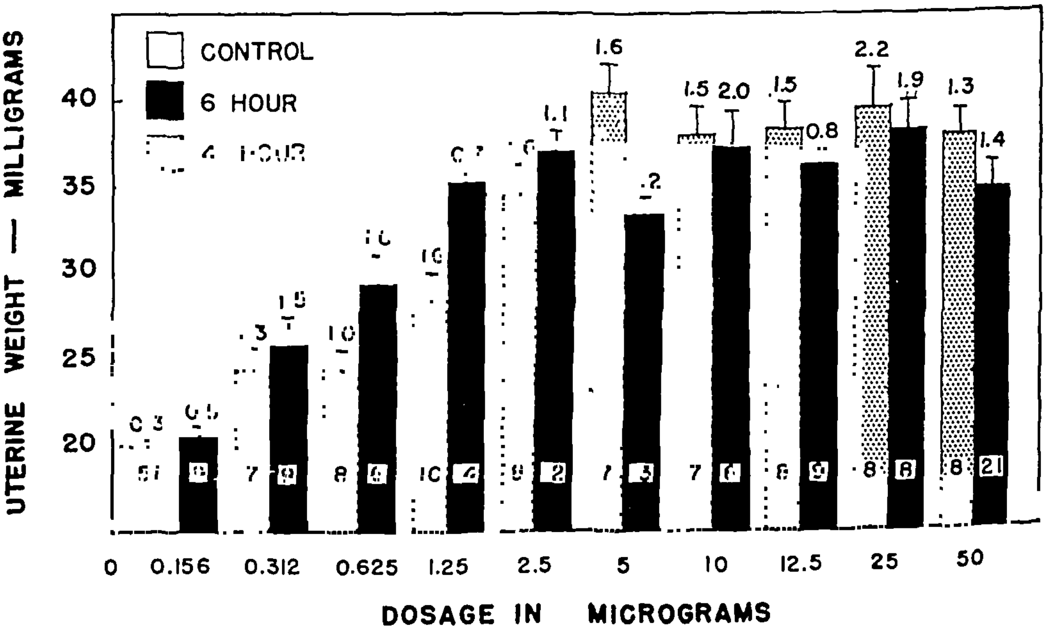

FIG. 4. Four and six hour response to equilin.

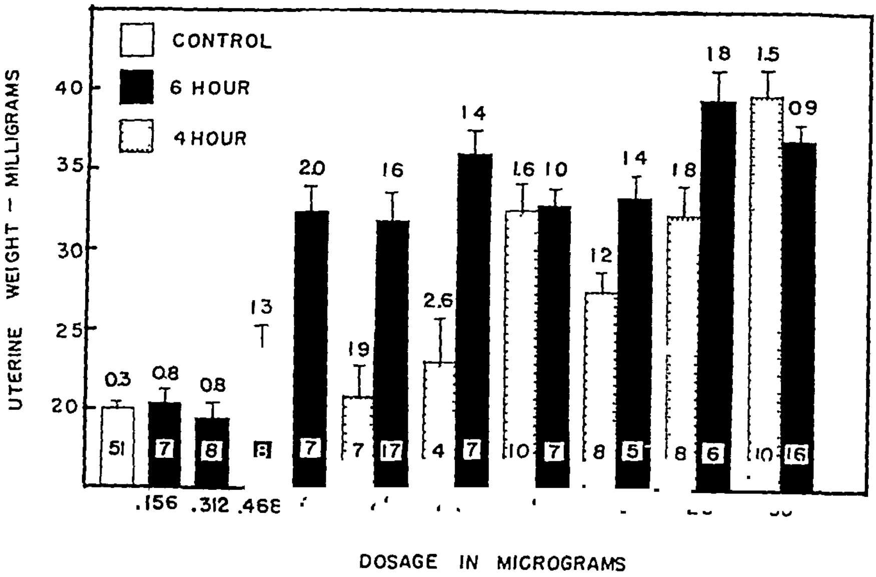

FIG. 5. Four and six hour response to equilenin.

The minimal dosage of estradiol producing a maximal response in six hours is 0.05 microgram. The uterine weight so obtained represents an increase of 75% over the control. The microgram amounts of the various estrogens required to produce a 70% increase, in their order of activity, are: *estradiol, 0.05; estriol, 0.078, diethylstilbestrol, 0.156; equilenin, 0.625;* estrone and equilin, 1.25. These results show that estradiol is 1½ times stronger than estriol, 3 times stronger than diethylstilbestrol, 12½ times stronger than equilenin, and 25 times more potent than estrone or equilin.

The dosage of an estrogen required to produce uterine weights at four

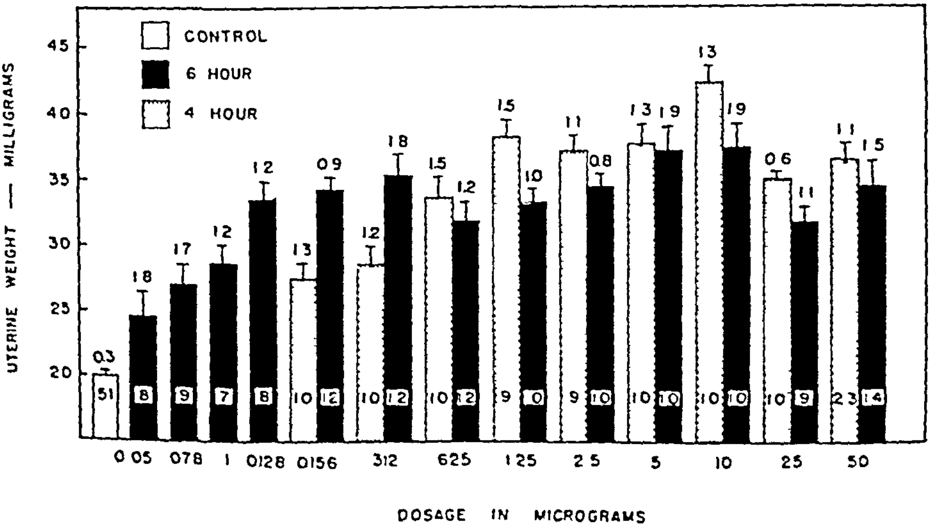

FIG. 6. Four and six hour response to diethylstilbestrol.

hours that are greater than those at six hours seems as characteristic for each compound as the amount required for an Astwood unit. A possible exception is estriol for which the four-hour response was first the greater at 0.039 µg. followed by considerable irregularity between the two responses as the dosage was increased (Fig. 1). With this possible exception, the minimal amounts of the different estrogens required to bring this about when listed in the order of strength in micrograms are as follows: estriol, 0.039; estradiol, 0.15; diethylstilbestrol, 0.625; equilin, 5; and equilenin and estrone, 50. It is seen that for this reaction estradiol is probably about one fourth as strong as estriol, four times stronger than diethylstilbestrol, 32 times stronger than equilin, and 320 times more potent than either equilenin or estrone.

Another comparison that can be made from the data obtained for these six estrogens is the greatest increase in uterine weight that each can induce in six hours regardless of dosage within the range used. When compared on this basis it is seen (Figs. 1–6) that the maximal weight attained by uteri on estradiol in this time was 35 mg. at a dosage of 2.5 µg. Approximately the same maximal weights were obtained for estrone at 2.5 µg. and diethylstilbestrol at 5 µg., while equilin at 10 µg. produced uteri somewhat larger. Estriol at 6.25 µg. and equilenin at 25 µg. are capable of producing uteri weighing more than forty milligrams which is at least a hundred per cent increase over the controls.

These observations indicate that if the early response to an estrogen is properly evaluated it is necessary to compare the dose-response curves for both four and six hours. This is well illustrated by estriol which at 50 µg. gives the same response as that for 0.156 µg. on the sixth hour. Examination of the four- and six-hour responses to corresponding intermediate doses of estriol shows they are quite different. The response to 0.156 µg. falls on the rising leg of the curve of fluid imbibition while that for 50 µg. is in the phase of water resorption having already reached a maximum at four hours. These effects as described for estriol are generally true for the other five estrogens although diethylstilbestrol seems to act more slowly in that a maximal response to a low dosage is reached at nine hours instead of six.

These observations show that fluid imbibition is not only a response common to all estrogens but that they differ widely in their ability to produce the effect. Also, the response to a particular estrogen is constant and characteristically related to time and dosage. On the basis of these differences, it can be stated that estradiol and estriol are approximately equal with respect to microgram amounts required to produce an Astwood unit, but three times more diethylstilbestrol, 12½ times more equilin, 18 times more estrone, and 21.8 times more equilenin is required to produce the same effect. However, for the production of 70% increase in uterine weights, estradiol is the most efficient, followed by estriol, diethylstilbestrol equilenin, estrone and equilin in that order. The data for the five estrogens also show that as the dosage is increased maximal imbibition occurs earlier, and that they differ with respect to the amount necessary to bring this about.

Uterine growth

Stimulation of uterine growth is a general property of estrogenic compounds, but some are more effective than others. Rapid growth begins between the ninth and twentieth hour in a typical response to a single injection of an estrogen, after which the rate progressively decreases as the uterus approaches its maximal response. Maximal uterine weights are usually attained by the fortieth hour followed by cessation of growth and involution, which begin at about the forty-eighth hour and are complete by the seventy-second hour at which time weights of the uteri approach those of untreated controls (Figs. 7–12).

The percentage of water in general reflects the physiological state of the uterus. During the first few hours fluid imbibition greatly increases and then falls rapidly during resorption, though it never reaches the control level, and again rises with the initiation of growth. When growth begins, as measured gravimetrically, water increases proportionate to uterine weight but never equals that of the first four or six hours during rapid fluid imbibition. Two exceptions to this general statement are the responses to equilin and diethylstilbestrol which at high doses continue the formation of luminal fluid in the presence of growth (Figs. 10 and 12).

Uterine dry weight is a reflection of uterine wet weight, as it too increases proportionately during growth. This is not due to solid material present in luminal fluid, but to tissue growth, as the dry weights of uteri from which the luminal fluid has been expressed are much greater than those of the controls. It is probably true that in the intact uterus during the growth phase a small portion of the dry weight may be due to solids of luminal fluid that have not been resorbed and certainly so when concurrent growth and imbibition occur such as that following injections of large doses of diethylstilbestrol or equilin. However, in general the uterine dry weights increase as growth progresses and decrease during the subsequent tissue involution.

Both high and low dosages of the estrogens were used for comparisons of growth responses following a single injection. The high dosage was in each instance fifty micrograms while the minimal amount of each compound required to give maximal water imbibition in six hours was used for the low dosage. The larger amount was selected arbitrarily while the smaller doses were based on a biological reaction and consequently probably should be considered as being more nearly physiological. However, the high dosages brought out certain growth characteristics that were not evident when small doses were given, for example, some estrogens were

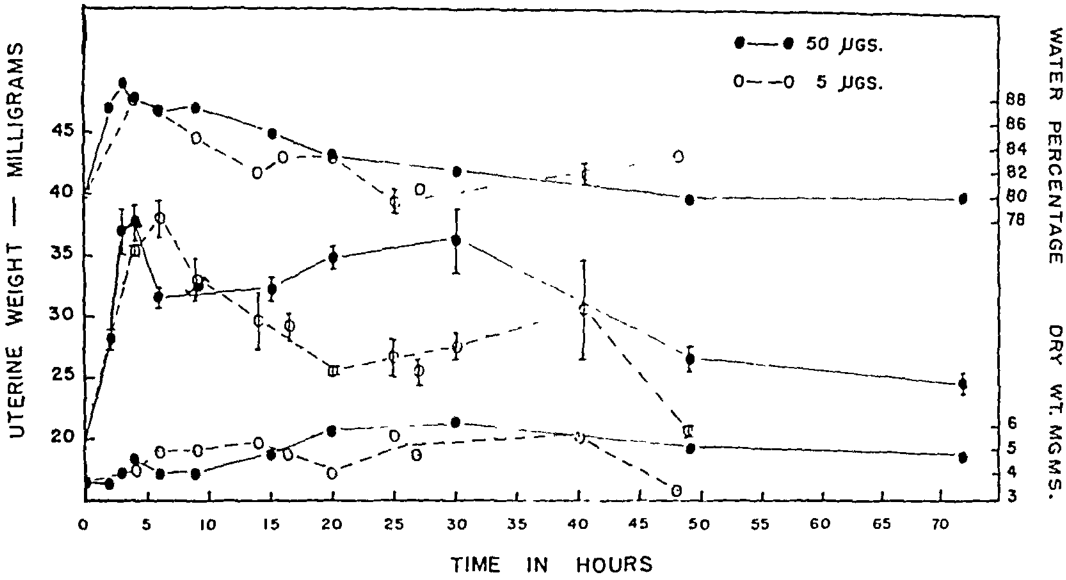

FIG. 7. Growth response with estriol. In figures 7 to 12 the vertical lines represent the standard error.

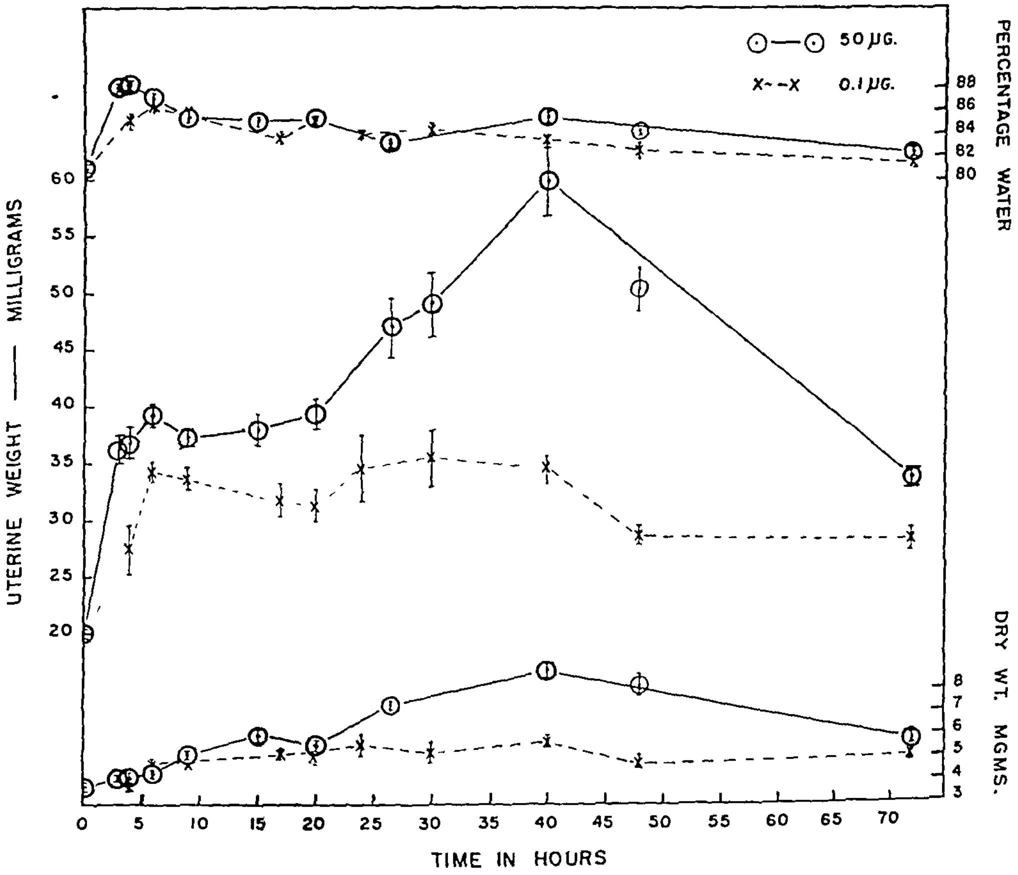

FIG. 8. Growth response with estradiol.

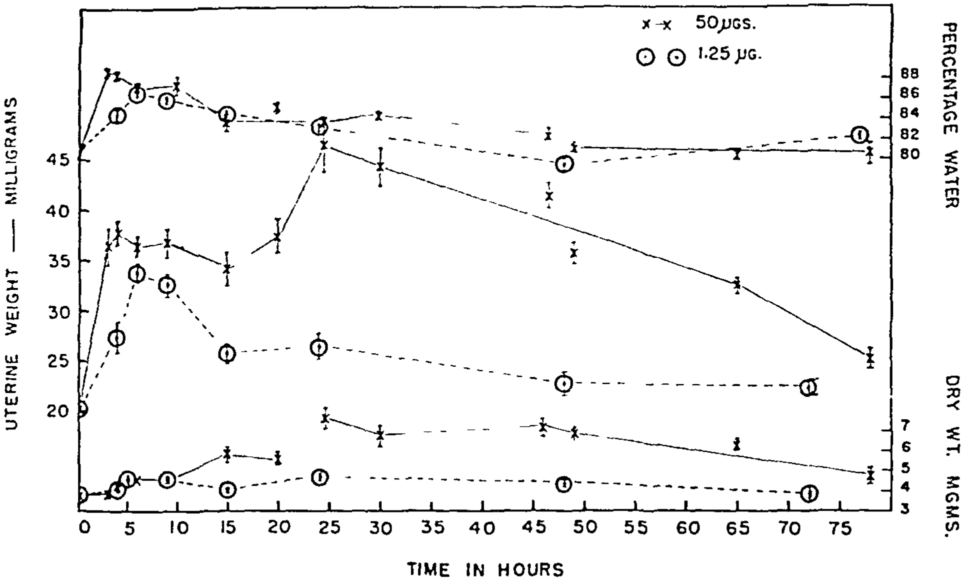

FIG. 9. Growth with estrone.

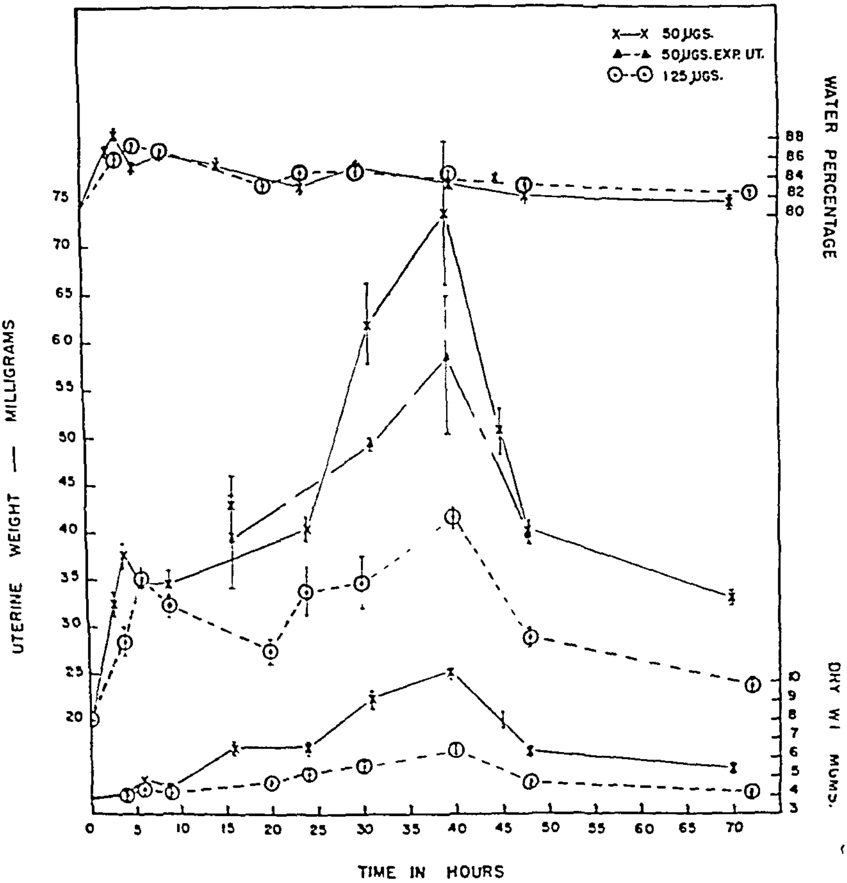

FIG. 10. Growth response with equilin.

found to be more active over longer periods of time than others. Also, the maximal growth with 50 µg. was greater for long acting estrogens, while those that acted for a shorter time produced their maximal effect earlier in the growth phase and the subsequent decline was more rapid (Figs. 7–12). These differences were due probably in part to difference in solubility of the steroids but this does not seem to offer a complete explanation.

Comparisons of growth produced by a dose of each estrogen based on the amount required to induce maximal fluid imbibition in six hours showed that these two effects are quite independent responses (Figs. 7–12).

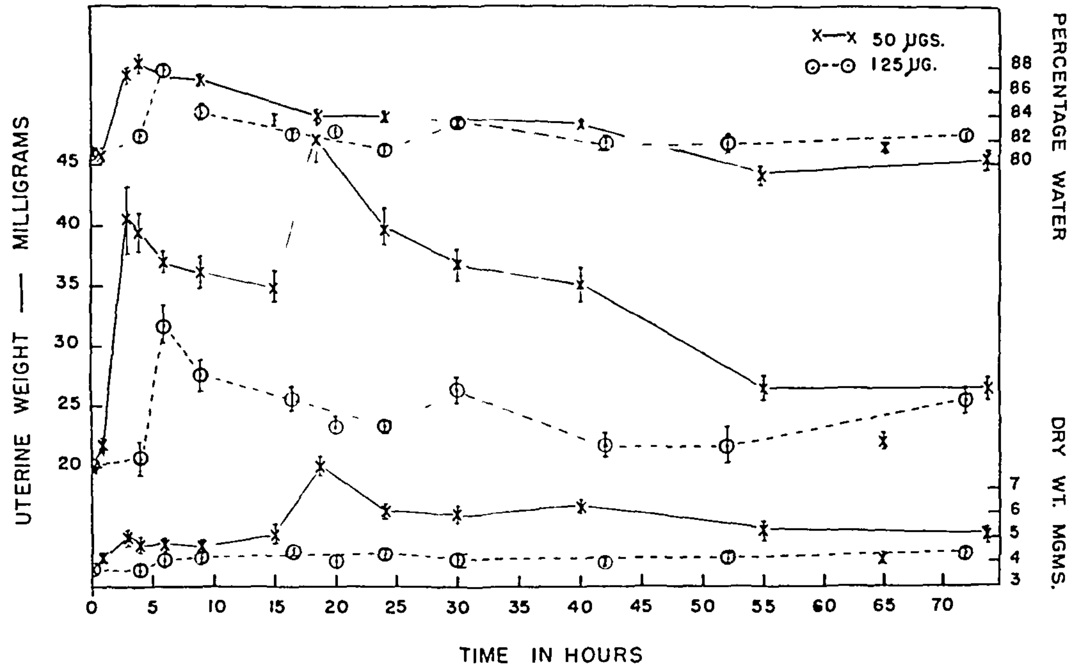

FIG. 11. Growth response with equilenin.

Some that are strong promoters of fluid imbibition are relatively weak for growth (equilenin and estriol) while others (estradiol and equilin) are equally effective for both. Equilenin and estriol in doses of 1.25 µg. give a good response for fluid imbibition at six hours (Figs. 1 and 5) and slight growth at 30 to 40 hours, while even 5 µg. estriol gives a poor growth response (Figs. 7 and 11).

The curves representing growth induced with fifty micrograms of the different estrogens (Figs. 7–12) show certain shifts in position with respect to each other during the growth phase. While the synthetic estrogen, diethylstilbestrol, is the strongest growth promoter and estriol is the weakest, the other four, estradiol, equilin, estrone, and equilenin, change their order of activity. Their relative positions at twenty hours in order of greatest activity are equilenin, estrone, estradiol, and equilin. However, when the respective growth curves are compared it is noted that equilenin, which ranks first at twenty hours, has already induced even greater uterine weights earlier, and its effect is in fact subsiding while at twenty-four hours equilenin has dropped to fifth place, being replaced by estrone. Equilin, at thirty and forty hours, is followed by estradiol, estrone (now subsiding), and equilenin. At forty-eight hours estradiol has replaced equilin, (now subsiding) otherwise the order remains the same as at forty hours. Diethylstilbestrol and equilin promote both uterine growth and fluid imbibition simultaneously, but even so such uteri after the fluid has been expressed, have wet weights at thirty and forty hours equal to those of animals given estradiol. The relative effectiveness of the six estrogens in

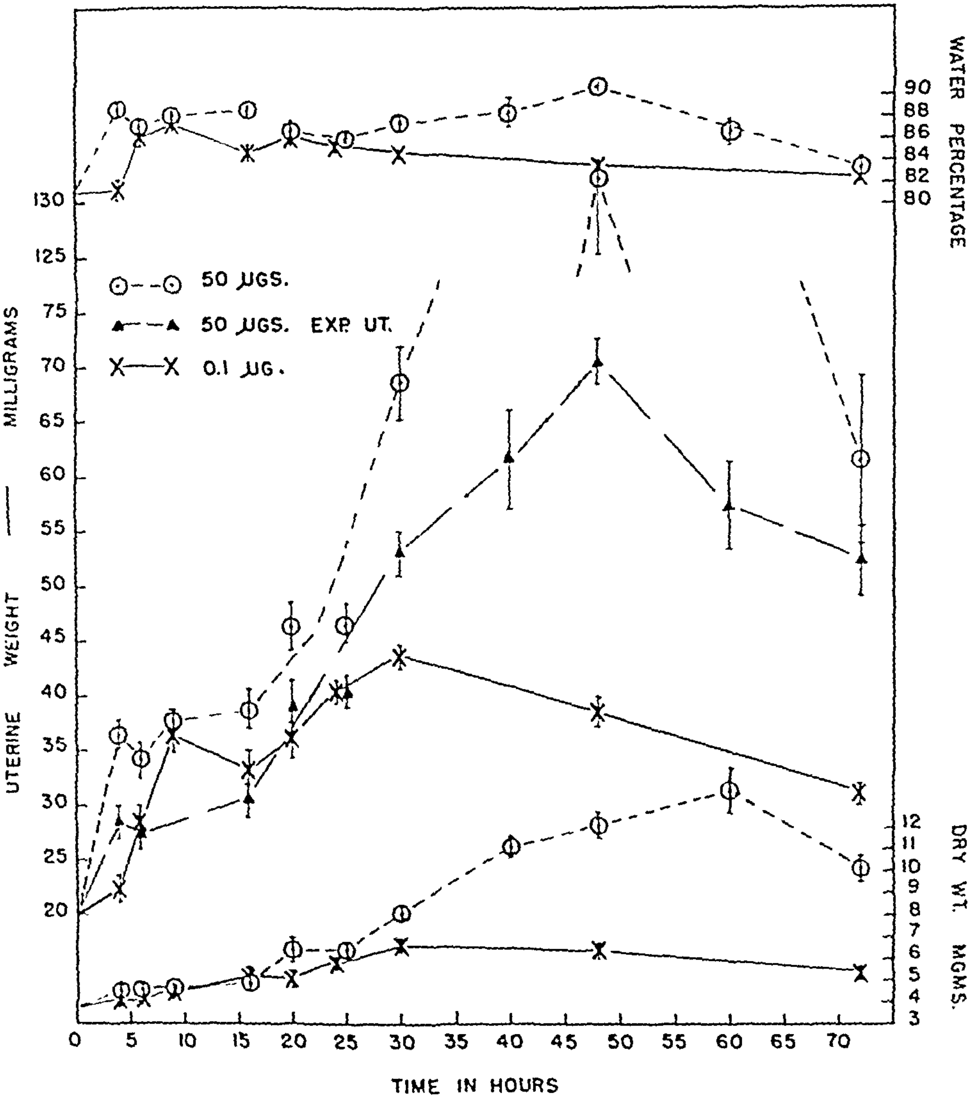

FIG. 12. Growth response with diethylstilbestrol.

order of their ability to stimulate over-all growth would appear to be diethylstilbestrol, estradiol, equilin, estrone, equilenin, and estriol, among which equilenin and estrone seem to act more rapidly than the others and consequently produce their effects at an earlier time.

These studies tend to show that while the action of estrogens are in general the same, each has its own individual peculiarities both as to dosage required for a particular reaction and the character and extent of the response. Although certain differences in potency and duration of action have been mentioned by many investigators these usually have been accredited to variation in rate of absorption, method of administration, rate of metabolic destruction and other factors rather than to the possibility of a physiological individuality of the compounds.

DISCUSSION

Many investigators have tested the relative activities of estrogens by various standardization procedures and their observations on uterine growth are in general agreement with our own. Also Szego and Roberts (9) found that in rats 24 to 27 days old the relative effectiveness of estrogens in promoting the uptake of water by the uterus was of the order estriol, estradiol-17B and estrone. However, a more detailed analysis of the action of estrogens on fluid imbibition and uterine growth has brought out more clearly certain individual diffferences.

It is obvious that certain variables need to be taken into consideration in evaluating such differences. Among these are variations in rate of absorption, rate of metabolic destruction, and methods of administration. In the present study all estrogens were given subcutaneously in 0.1 ml. sesame oil. There was little difference in character of the response in these experiments and others in which 0.1 ml. of propylene glycol was used. Szego and Roberts (9) found that imbibition of uterine fluid in young rats on the sixth hour was in general the same when estrogens were given intraperitoneally either in 0.2 ml. oil or as sodium salts in physiological saline. Apparently the most important difference was a three-fold increase in the response to estriol when given in saline while estradiol and estrone were equally effective in both. The order of effectiveness remained the same, being estriol, estradiol, and estrone which is in agreement with our observations. We have found that the estrogens when listed in order of their ability to produce approximately 70% increase in uterine wet weight by the sixth hour are estriol, estradiol, diethylstilbestrol, equilenin, estrone and equilin. Though much is yet unknown regarding the nature of estrogenic action, our data indicate that estrogens may differ both as to the amount required for a particular reaction and as to the character of the response. Some are more effective for imbibition of uterine fluid while others are more active in the promotion of uterine growth and superior capacity for one does not necessarily indicate superior capacity for the other. For example, estradiol is the strongest of the natural estrogens for growth and excels at low doses for fluid imbibition while equilin ranks second to estradiol at all doses for growth, is weak at low doses for imbibition, but is very effective for both growth and imbibition at high doses.

As to the physiological meaning of these differences, nothing definite is known but it may be significant that ordinarily two or more estrogens are present and presumably act concurrently in the promotion of a response. Estradiol-17B and estrone are apparently hormones of the estrous cycle, while in women estradiol-17B and estrone and a small amount of estriol are present during the menstrual cycle (4), and in pregnancy, estriol becomes by far the dominant hormone (5, 6). A similar situation seems to hold for the mare. Estradiol-17B and estrone are found during the estrous cycle while in pregnancy at least three additional steroids appear; equilin, equilenin and dihydro-equilenin, of which the first two are the most abundant (7). The observations in the present study on two estrogen effects, fluid imbibition and growth, suggest the possibility that under normal conditions the combined effect of the several estrogens may be one in which some are more effective than others for certain aspects of the reaction or that the action of one may have been limited by that of another (10, 11). Also, the abundance of estriol in women and equilin and equilenin in mares during pregnancy strongly suggests that these steroids are hormones of pregnancy and the differences in activity reported in this study seem to support the thought that they may indicate adaptations for the performance of physiological processes peculiar to gestation.

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NOTES AND COMMENTS

DEPRESSION OF THE MOUSE THYROID IODIDE PUMP BY GOITROGENS

ABSTRACT

The administration of thiouracil or propylthiouracil to mice maintained on a moderately low iodide diet results in depression of the activity of the iodide pump per unit of thyroid weight (expressed as the thyroid/serum radioiodide gradient, T/S. The activity of the pump per gland (T/S) increases with increase in total thyroid weight, increase in T/S or increase in both.

Several groups (1, 2, 3) have reported that thyroid iodide pump activity (T/S) of rats is increased after the administration of thiouracil (TU) or propylthiouracil (PTU). Lipner *et al.* (4) and Scow and Greer (5) have observed that the administration of PTU to mice decreases the T/S. This report presents the data on which the abstract of Lipner *et al.* (4) was based.

METHODS

Thirty-five C_3H-HeN male mice three months old were randomized into seven groups and placed on an adequate stock diet of natural foodstuffs moderately low in iodine.[1] To this diet were added varying amounts of either PTU or TU (Table 1), and the animals were fed their respective diets until the experiment was terminated at the end of four weeks.

TABLE 1. DEPRESSION OF THE THYROID IODIDE PUMP BY GOITROGENS

Treatment	Mouse wt., gm.	Thyroid weight	T/S	t/S	Mean acinar cell height
Control	24.4 ±0.8	1.70 ±0.12*(4)†	197.3 ±11.8	3.35	9.5 ±0.3
1250 µg. TU‡	23.8 ±0.3	16.72 ±2.07 (5)	119.6 ± 2.5	20.0	14.9 ±0.4
2500 µg. TU	20.4 ±0.8	18.60 ±2.0 (4)	146.6 ±11.0	27.3	13.7 ±0.3
5000 µg. TU	22.9 ±1.0	11.96 ±1.08 (5)	191.1 ±15.6	22.9	14.6 ±0.3
125 µg. PTU§	23.0 ±1.6	13.88 ±2.06 (5)	119.6 ±13.2	16.5	17.5 ±0.4
250 µg. PTU	23.9 ±0.8	17.46 ±2.24 (5)	132.8 ± 6.5	23.2	16.9 ±0.3
500 µg. PTU	23.4 ±0.6	15.30 ±2.26 (5)	168.9 ± 9.3	25.1	16.8 ±0.2

$$* \text{ Standard Error of the Mean} = \frac{\text{range}}{\text{No. of Animals}} \quad (8)$$

† Number of animals.
‡ Thiouracil/gm. diet.
§ Propylthiouracil/gm. diet.

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[1] The composition of the Morris moderately low iodide diet No. 77 is as follows:

Skim milk	22.75%
Ground whole wheat	61.25%
Dehydrated brewer's yeast	4.00%
NaCl	1.40%
Ferric citrate	0.13%
Cod liver oil	0.50%
Corn oil	9.70%
	100.00%

A modified version of this diet No. 77-A in which the cod-liver oil was replaced by a vitamin A and D concentration in corn oil contained by analysis 0.75 ±0.039 µg. of iodine/gm. of diet (6).

The T/S was determined as described by Halmi *et al.* (3). Forty-five minutes after the acute blocking dose of 1.0 mg. PTU per mouse, the mice received 1 μc of I^{131} injected subcutaneously on the back in the region over the pelvis. The animals were killed between 60 and 90 minutes after the I^{131} was administered. The planchet technic of VanderLaan and Greer (2) for determining the I^{131} content of thyroids and serum was used. The radioactivity was determined by means of a Geiger-Muller counter. The T/S was calculated from the ratio of radioiodide per 100 mg. thyroid tissue to the radioiodide per 0.1 ml. serum. The t/S was calculated from the ratio of radioiodide per total gland to the radioiodide per 0.1 ml. of serum (6) or t/S = T/S × total gland weight/100.

The thyroids were dissected under a binocular microscope at 9× magnification and weighed immediately on a Roller-Smith torsion balance. Radioactivity was determined on one lobe. The other lobe was fixed in SUSA, embedded in paraffin, and sectioned at 5μ for histological examination. One cell was selected for cell height measurement from each acinus that appeared to be sectioned through the center. The sections were projected on a screen and in each section the acinar height of 20 cells was measured.

RESULTS AND DISCUSSION

The data in Table 1 indicate that PTU and TU had an effect on mouse thyroid weight and acinar cell height similar to that seen in the rat. The effect of these drugs on mouse thyroid function when the T/S is used as the measure was dissimilar. Compared with the controls both PTU and TU depressed the T/S at low dose levels. At the highest dose level TU was less effective than the lower doses in stimulating thyroid growth and there was no depression of T/S. PTU caused a progressive increase in T/S as the dose increased.

Halmi (7) has suggested that the t/S is a more reliable index of iodide pump activity than the T/S especially when gland weights are greatly different. The t/S calculation applied to the data of Seow and Greer (5) indicates that the iodide pump activity per gland increases with increased gland weight (7). Table 1 shows the t/S and T/S for the present data. In the mouse the mechanism responsible for iodide accumulation does not increase at the same rate that thyroid weight increases. As a result T/S decreases while t/S rises. From the point of view of the whole animal, the t/S is a more meaningful index of thyroid function than is the T/S which reflects the functional status of a unit quantity of the gland.

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[3] This investigation was supported by a postdoctoral fellowship from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare.

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NOTES AND COMMENTS

DEPRESSION OF THE MOUSE THYROID IODIDE PUMP BY GOITROGENS

ABSTRACT

The administration of thiouracil or propylthiouracil to mice maintained on a moderately low iodide diet results in depression of the activity of the iodide pump per unit of thyroid weight (expressed as the thyroid/serum radioiodide gradient, T/S. The activity of the pump per gland (T/S) increases with increase in total thyroid weight, increase in T/S or increase in both.

Several groups (1, 2, 3) have reported that thyroid iodide pump activity (T/S) of rats is increased *after the administration of thiouracil (TU) or propylthiouracil (PTU)*. Lipner *et al.* (4) and Scow and Greer (5) have observed that the administration of PTU to mice decreases the T/S. This report presents the data on which the abstract of Lipner *et al.* (4) was based.

METHODS

Thirty-five C_3H-HeN male mice three months old were randomized into seven groups and placed on an adequate stock diet of natural foodstuffs moderately low in iodine.[1] To this diet were added varying amounts of either PTU or TU (Table 1), and the animals were fed their respective diets until the experiment was terminated at the end of four weeks.

TABLE 1. DEPRESSION OF THE THYROID IODIDE PUMP BY GOITROGENS

Treatment	Mouse wt., gm.	Thyroid weight	T/S	t/S	Mean acinar cell height
Control	24.4 ±0.8	1.70 ±0.12*(4)†	197.3 ±11.8	3.35	9.5 ±0.3
1250 μg. TU‡	23.8 ±0.3	16.72 ±2.07 (5)	119.6 ± 2.5	20.0	14.9 ±0.4
2500 μg. TU	20.4 ±0.8	18.60 ±2.0 (4)	146.6 ±11.0	27.3	13.7 ±0.3
5000 μg. TU	22.9 ±1.0	11.96 ±1.08 (5)	191.1 ±15.6	22.9	14.6 ±0.3
125 μg. PTU§	23.0 ±1.6	13.88 ±2.06 (5)	119.6 ±13.2	16.5	17.5 ±0.4
250 μg. PTU	23.9 ±0.8	17.46 ±2.24 (5)	132.8 ± 6.5	23.2	16.9 ±0.3
500 μg. PTU	23.4 ±0.6	15.30 ±2.26 (5)	168.9 ± 9.3	25.1	16.8 ±0.2

* Standard Error of the Mean $= \dfrac{\text{range}}{\text{No. of Animals}}$ (8)

† Number of animals.

‡ Thiouracil/gm. diet.

§ Propylthiouracil/gm. diet.

Received January 24, 1958.

[1] The composition of the Morris moderately low iodide diet No. 77 is as follows:

Skim milk	22.75%
Ground whole wheat	61.25%
Dehydrated brewer's yeast	4.00%
NaCl	1.40%
Ferric citrate	0.13%
Cod liver oil	0.50%
Corn oil	9.70%
	100.00%

A modified version of this diet No. 77-A in which the cod-liver oil was replaced by a vitamin A and D concentration in corn oil contained by analysis 0.75 ±0.039 μg. of iodine/gm. of diet (6).

The T/S was determined as described by Halmi *et al.* (3). Forty-five minutes after the acute blocking dose of 1.0 mg. PTU per mouse, the mice received 1 μc of I^{131} injected subcutaneously on the back in the region over the pelvis. The animals were killed between 60 and 90 minutes after the I^{131} was administered. The planchet technic of VanderLaan and Greer (2) for determining the I^{131} content of thyroids and serum was used. The radioactivity was determined by means of a Geiger-Muller counter. The T/S was calculated from the ratio of radioiodide per 100 mg. thyroid tissue to the radioiodide per 0.1 ml. serum. The t/S was calculated from the ratio of radioiodide per total gland to the radioiodide per 0.1 ml. of serum (6) or t/S = T/S × total gland weight/100.

The thyroids were dissected under a binocular microscope at 9× magnification and weighed immediately on a Roller-Smith torsion balance. Radioactivity was determined on one lobe. The other lobe was fixed in SUSA, embedded in paraffin, and sectioned at 5μ for histological examination. One cell was selected for cell height measurement from each acinus that appeared to be sectioned through the center. The sections were projected on a screen and in each section the acinar height of 20 cells was measured.

RESULTS AND DISCUSSION

The data in Table 1 indicate that PTU and TU had an effect on mouse thyroid weight and acinar cell height similar to that seen in the rat. The effect of these drugs on mouse thyroid function when the T/S is used as the measure was dissimilar. Compared with the controls both PTU and TU depressed the T/S at low dose levels. At the highest dose level TU was less effective than the lower doses in stimulating thyroid growth and there was no depression of T/S. PTU caused a progressive increase in T/S as the dose increased.

Halmi (7) has suggested that the t/S is a more reliable index of iodide pump activity than the T/S especially when gland weights are greatly different. The t/S calculation applied to the data of Scow and Greer (5) indicates that the iodide pump activity per gland increases with increased gland weight (7). Table 1 shows the t/S and T/S for the present data. In the mouse the mechanism responsible for iodide accumulation does not increase at the same rate that thyroid weight increases. As a result T/S decreases while t/S rises. From the point of view of the whole animal, the t/S is a more meaningful index of thyroid function than is the T/S which reflects the functional status of a unit quantity of the gland.

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[2] Division of Physiology, Florida State University, Tallahassee, Florida.

[3] This investigation was supported by a postdoctoral fellowship from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare.

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LIMB REGENERATION IN THE SALAMANDER, *TRITURUS VIRIDESCENS*, AFTER LARGE INITIAL AND DURING PROLONGED SMALLER INJECTIONS OF CORTISONE ACETATE[1]

ABSTRACT

A study of the effect of cortisone on limb regeneration was made with the salamander, *Triturus viridescens*. One group of 50 animals was given a large initial dose of 4000 μg of cortisone acetate at the time of amputation of the left foreleg. A second group of 50 animals was given small doses of cortisone acetate over a period 3 to 4 weeks at the rate of 1000 μg per injection every two days until a total dosage of 12,000 μg was attained.

Blastema formation was delayed from 2 to 6 days after which regeneration was normal in comparison to the controls. Neither epithelium nor connective tissue growth were inhibited after the formation of the blastema.

The inhibition of growth of connective tissue in general by cortisone has been demonstrated by numerous investigators, and its selectivity for mesenchymal tissue acknowledged. The study of wound healing following mechanical injury to the skin in cortisone treated laboratory animals was the method of investigation most frequently used (1, 2, 3). Local thinning of the epidermis and dermal connective tissue results following applications of cortisone to areas of the rat neck (4, 5, 6). Parenteral administration of adrenocorticotropin also causes thinning of the dermal connective tissue (7).

This study was undertaken to determine the effect of cortisone on limb regeneration in the salamander. The fact that this investigation is concerned with a cold-blooded vertebrate should not be ignored. It is not to be assumed that results obtained by research on mammals can necessarily be extrapolated directly to the cold-blooded vertebrates.

MATERIAL AND METHODS

Adult *Triturus viridescens* maintained in the laboratory in a specially designed aquarium (8), were employed in this study. The experimental animals were divided into two groups of 50 adult animals each. In addition there were 25 control animals in each experiment.

The first group were given a large initial dose of cortisone acetate at the time of amputation of the left foreleg. The amount of the dose was 4 mg. (4000 μg.) in a saline suspension. Twenty-five controls were given only the saline base.

The second group received a total dosage of 12 mg. (12,000 μg.) of cortisone acetate over a period of 3 to 4 weeks after amputation of the left foreleg. One mg. was given every two days. The 25 controls received only the saline base in the same amount and at the same time as the experimentals. All injections were given intraperitoneally.

The regenerates studied histologically were fixed in 10% formalin and serially sectioned at 8 microns. The sections were stained with Mallory's stain.

RESULTS

In the first group four animals died a few days after the injection. About 75% of the remaining 46 animals showed a retardation in the formation of the blastema as compared to the controls. This retardation varied from 3 to 6 days. After 4 to 6 weeks no differences could be detected between the size of the regenerates of the experimental and control animals.

Although there was a delay of 3–6 days in blastema formation of experimentals in comparison to the controls, regeneration proceeded normally, and the resulting regenerates were equal in size to those of the controls. Histological studies did not reveal any suppression of connective tissue growth.

Several experimental animals in the second group showed an early necrosis of the stump, but all were normal in a few weeks. About 50% of the experimental animals showed a delay of about 2 to 3 days in formation of the blastema. No difference could be detected after 6 weeks in the regenerating capacities of the experimental animals in

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[1] Supplied through the courtesy of Merck & Co., Inc.

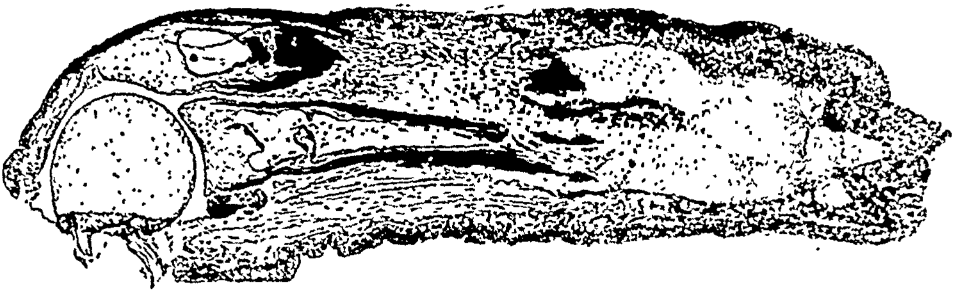


FIG. 1

comparison with the controls. Cortisone failed to suppress the growth of connective or epithelial tissues as was the case in the first experiment.

DISCUSSION

The results of this study indicate that cortisone does not inhibit growth of amphibian tissue as depicted by regeneration in the salamander. Comparable results were obtained by Manner (9) with a small daily injection of cortisone acetate (0.005 mg.). His study was concentrated on the early stages of regeneration, and reported a delay in blastema formation of about 4 days.

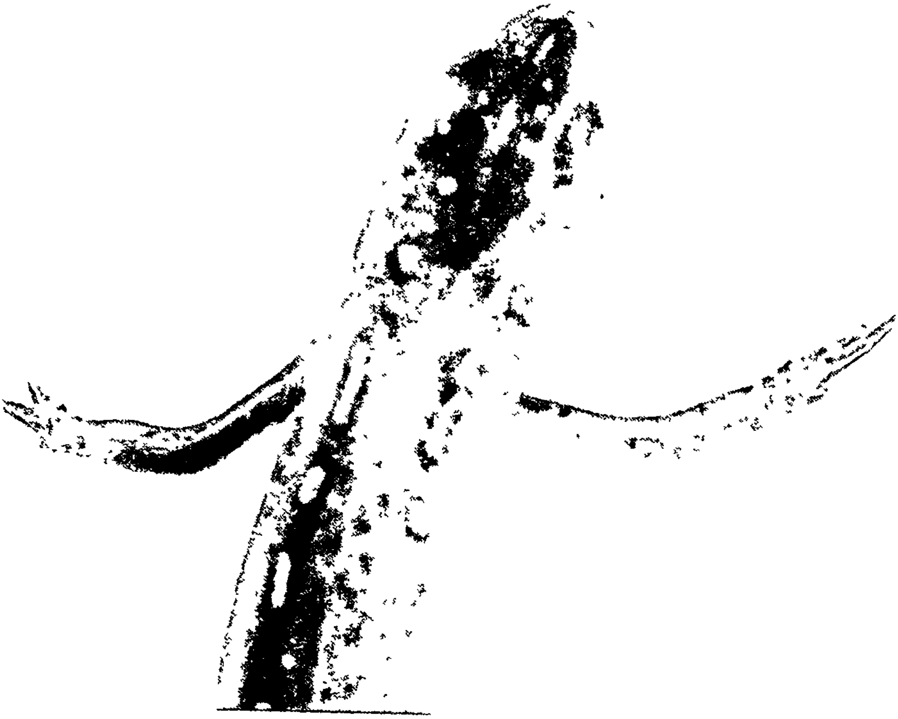


FIG. 2

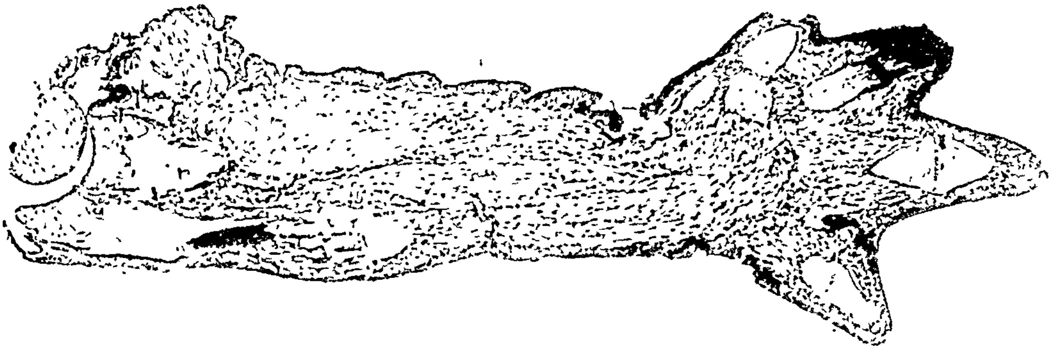
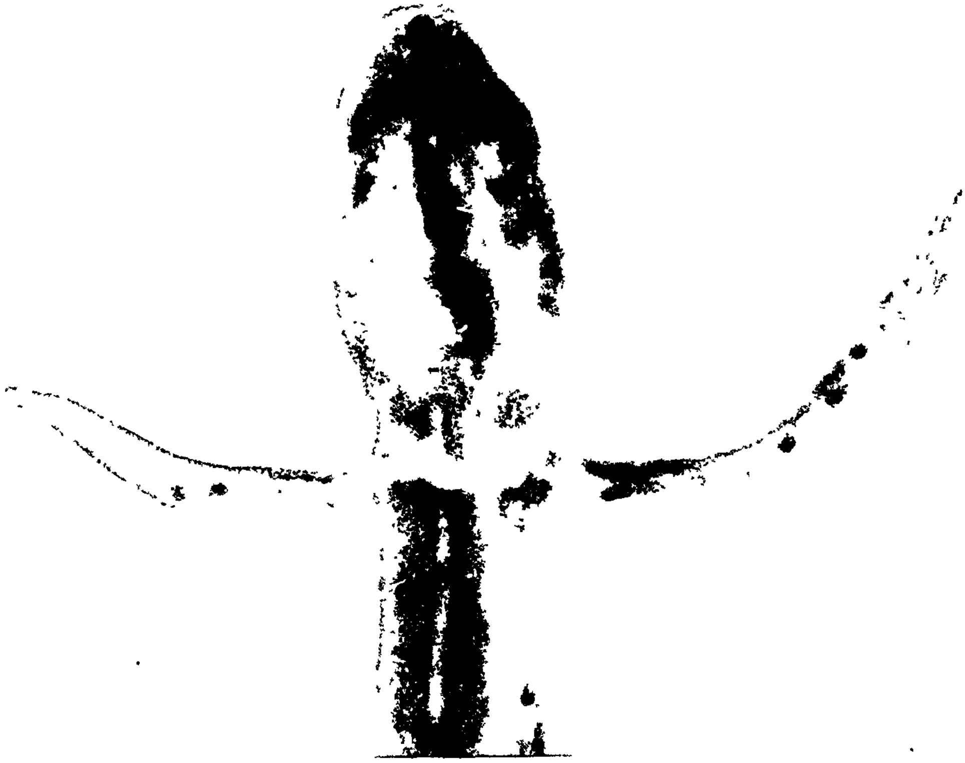


Fig. 3



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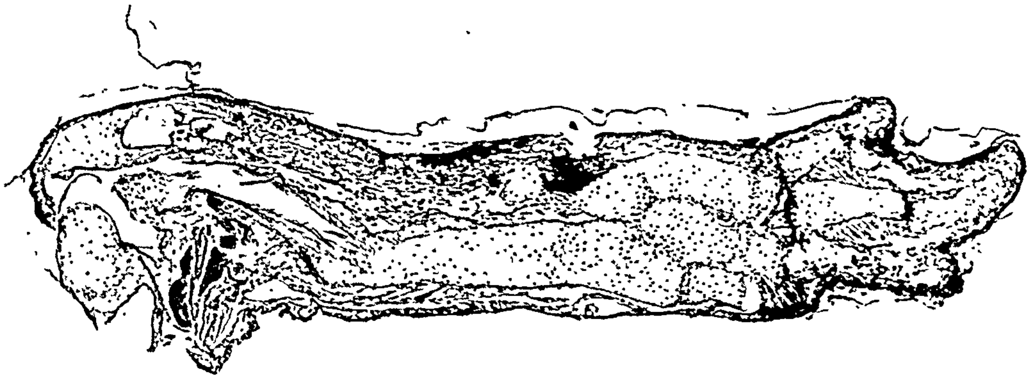


Fig. 5

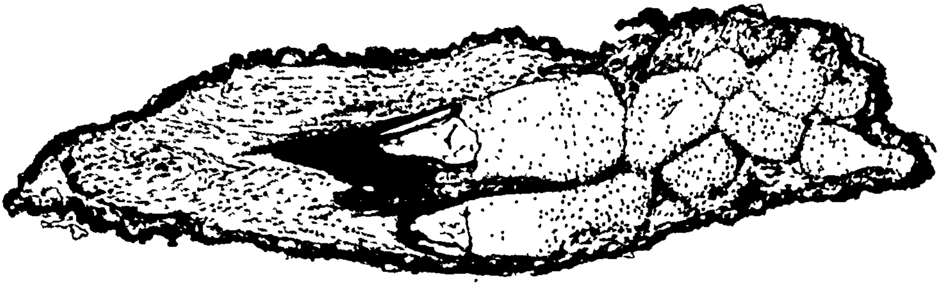
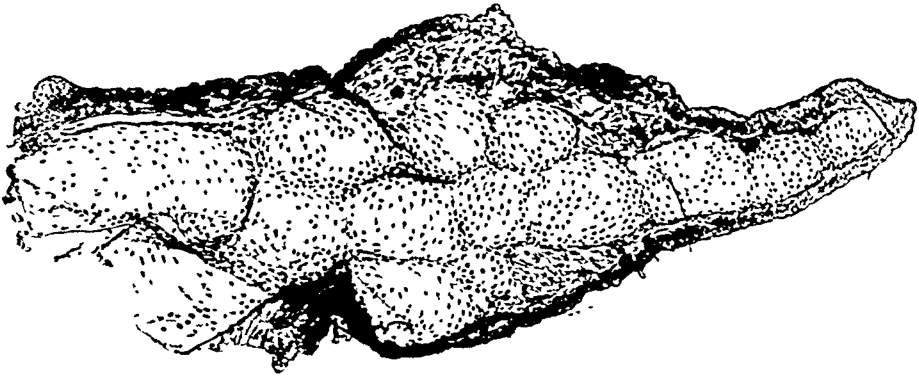


FIG. 6



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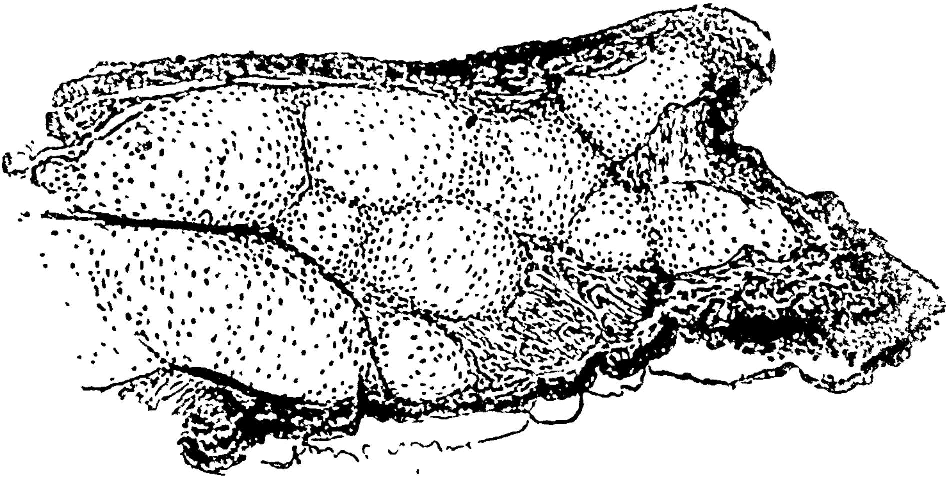

FIG. 8

The present study indicates that the salamander will tolerate a high dosage of cortisone without untoward effects on its regenerative capacities. There is, however, a thinning of the epidermis in the wound area (9) which does not persist after blastema formation, even though injections may be continued beyond that point.

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THE INFLUENCE OF GELATIN IN THE ASSAY OF PURIFIED PARATHYROID EXTRACTS

ABSTRACT

Parathyroid extracts purified by solvent distribution appeared to have lost activity when assayed in calcium-depleted, parathyroidectomized rats. The addition of gelatin as a vehicle to these extracts almost completely restored their original potency in a 4-hour test; there was little effect at 8, and none at 24 hours. When gelatin was added to Parathyroid Extract, U.S.P. (Lilly), no increase in potency was observed.

The potentiating effect of the vehicle with purified preparations might be explained by delayed absorption from the injection site.

During studies on the fractionation of parathyroid extracts it was found that activity seemed to be lost during the course of purification. Previous observations with purified corticotropin (1) suggested that rate of absorption might be a factor and that the addition of gelatin as a vehicle might increase the apparent potency of the purer materials. Moreover, Rasmussen and Westall, using a different assay method, reported a shortened duration of action of a partially purified preparation (2). They further found that the activity of their material was prolonged by suspension in arachis oil. This report concerns the influence of gelatin as a vehicle in the assay of partially purified parathyroid hormone.

MATERIALS AND METHODS

The extract used in the experiments reported here was prepared by butanol extraction of Parathyroid Extract U.S.P. (Lilly) dissolved in 1% NaCl-6% acetic acid (3). After adding one-half volume of water the butanol was removed under reduced pressure at 60° C in a rotatory evaporator. The activity was then precipitated from aqueous solution with 2%

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Table 1

	Parathyroid extract, U.S.P.	Parathyroid extract, U.S.P. plus gelatin
Group	A	G
Number animals	15	15
Average log dose (12.5 U.S.P. units)	1.0969	1.0969
Average response Serum Ca (mg./100 ml.)	10.27	10.07
Slope	3.1157	2.8895
Combined slope		3.0026
Potency of G compared to A		86% (50–210% at the 95% significance level)

trichloroacetic acid. Following conversion to the acetate form with an ion-exchange resin, the material was diluted with weak acetic acid to the appropriate concentration for assay. Gelatin, when used, was added to the assay dilutions as an equal volume of a 30% solution. All fractions were administered subcutaneously at equal final concentration of activity in 0.2 to 0.8 ml. volumes. A modification of the bioassay of Munson (4) was used: eighty-gram male rats were fed a calcium-deficient diet for four days and then parathyroidectomized and injected immediately with the assay material. The small variation of body weight of the rats was neglected. Samples of sera obtained four hours after parathyroidectomy were assayed for calcium using alizarin (5). Parathyroid Extract, U.S.P., was used as the bioassay standard in all cases. Results of assays were analyzed according to Irwin (6).

RESULTS

A three-dose comparative assay of Parathyroid Extract, U.S.P., in gelatin solution against the same material without gelatin showed no significant difference in potency (Table 1). However, the vehicle greatly enhanced the effect of the material prepared by butanol extraction. Figure 1 represents the results of a two-dose assay comparing this partially purified substance with and without gelatin against the standard in gelatin. The diminished responses and slope produced by the gelatin-free preparation fell below the assay range. Thus, these results could not be compared on a bioassay basis, but a "t" test showed the difference in response between the purified preparation alone and

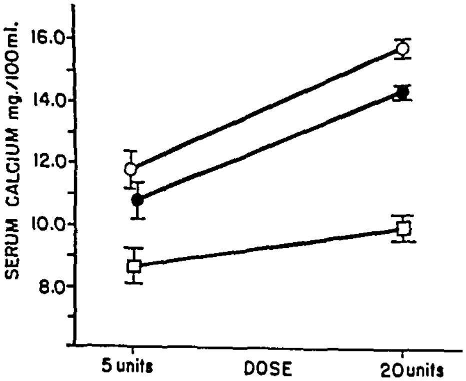

Fig. 1. Two-dose assay comparing partially purified parathyroid extract alone □—□, and in gelatin ●—●, vs. Parathyroid Extract, U.S.P., in gelatin ○—○. Each point represents the mean value (±S. E.) obtained from 5 animals.

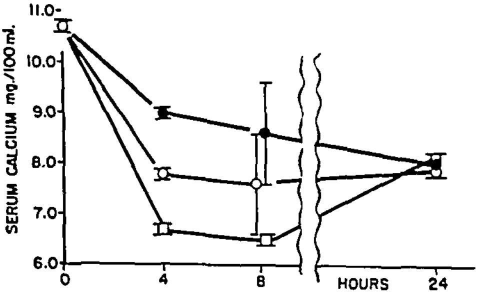

Fig. 2. Concentration of calcium in sera of rats parathyroidectomized at time zero and injected immediately with partially purified extract alone ○—○, or with gelatin added ●—●. The points represent the mean value (±S. E.) using 5 or 6 rats per group. The control group □—□, was parathyroidectomized, but not injected.

the purified preparation in gelatin to be highly significant ($p = <.05$ for the 5 unit dose; $p = <.001$ for the 20 unit dose).

The influence of gelatin as a vehicle on the ability of this preparation to prevent the fall in concentration of calcium in the serum of the assay animals is shown in Figure 2. Each injected rat received the equivalent (approximately 0.2 mg. dry weight) of 20 units of the starting material. The animals were allowed a normal diet after eight hours in an attempt to prevent death from hypocalcemia. Nevertheless, two of the five control animals died before eight hours, and a third before twenty-four hours following parathyroidectomy. Addition of the vehicle to the extract caused a significant difference in the concentration of calcium in the sera four hours following parathyroidectomy and injection ($p = <.05$), but at eight hours and beyond the differences were not significant.

DISCUSSION

The addition of gelatin as a vehicle almost completely restored the apparent loss of activity of the material studied here. A similar effect has also been found when assaying fractions prepared by paper chromatography. These observations, in addition to those of Rasmussen and Westall, suggest that admixture with appropriate vehicles is necessary in following the course of purification of this hormone.

The mechanism through which gelatin potentiated the action of partially purified extracts was not elucidated. Perhaps the enhanced effect was due to slower release of the hormone from subcutaneous tissues. Such an explanation is supported by Munson's conclusion that the hormone has a short life *in vivo* (4). The large amount of inert protein in Parathyroid Extract, U.S.P., may act in a similar manner. Thus, when gelatin was added to that material, no increase in potency was observed. However, prevention of inactivation in dilute solutions, or prevention of adsorption to glass, might have contributed to the observed effect.

Preparations of higher purity isolated by countercurrent distribution sustained greater losses of activity which were not completely restored by the adjuvant (3). The cause of this further apparent inactivation was not found.

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PREVENTION OF ADRENAL-REGENERATION HYPERTENSION BY AN ALDOSTERONE-BLOCKER

ABSTRACT

Chronic treatment of rats with the aldosterone-blocker 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone prevented adrenal-regeneration hypertension and the accompanying cardiac hypertrophy. Unaffected were: mortality, body weight, saline intake, renal and adrenal hypertrophy.

A steroidal lactone was recently described (1) that was found to antagonize the renal excretory effects of mineralocorticoids in laboratory animals (2, 3) and man (4). This compound was also found to have a hypotensive action in metacorticoid hypertensive rats and to prevent the pressor action of desoxycorticosterone acetate in saline-fed rats (5, 6). It was therefore of interest to determine the ability of the steroid to prevent the induction of adrenal-regeneration hypertension in rats, a disease that may be due to hyperreactivity to aldosterone (7). This condition can be prevented or reversed by adrenal depression with Amphenone (8).

MATERIAL AND METHODS

Twenty-four, female, Sprague-Dawley rats, 26 days old, were subjected to a one-stage right adrenalectomy, right nephrectomy, and left adrenal enucleation, following Skelton's procedure (9). They were offered Rockland Rat Diet and 0.86% sodium chloride solution *ad libitum* and, 2 days post-operatively, half of the rats received subcutaneous injections of 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (SC-5233) and the other half received equivalent volumes of the propylene glycol solvent. SC-5233 dosage was 20 mg./kg. twice a day on weekdays and 40 mg./kg. once a day on weekends for 8 weeks. Once a week, blood pressures (10), body weights, and fluid intakes were measured; the whole experiment was conducted in a rigidly-controlled "blind" fashion (11). Statistical procedures were based on the rank-sum test (12); standard errors are listed only to indicate degree of variability.

RESULTS AND DISCUSSION

Chronic treatment with SC-5233 prevented the rise in blood pressure and the cardiac hypertrophy without appreciably affecting mortality, body weight, spontaneous saline intake, or renal and adrenal hypertrophy (Tables 1, 2). It is tempting to postulate that

TABLE 1

	Weeks of treatment							
SC-5233 group	1	2	3	4	5	6	7	8
No. rats	12	12	12	12	11	11	10	9
Blood press., mm.	148 ± 2	142 ± 2*	152 ± 2†	159 ± 1†	159 ± 1§	160 ± 2†	153 ± 3	153 ± 2§
Body wt., gm.	106 ± 3	133 ± 2	160 ± 2	179 ± 4	182 ± 7	183 ± 7	213 ± 11	217 ± 10
NaCl intake, ml./rat/day	45 ± 3	62 ± 4	67 ± 4	64 ± 4	64 ± 4	60 ± 7	70 ± 6	59 ± 5†
Placebo group								
No. rats	12	12	12	12	12	11	10	9
Blood press., mm.	152 ± 2	149 ± 2	161 ± 2	165 ± 2	171 ± 2	175 ± 1	166 ± 2	170 ± 1
Body wt., gm.	107 ± 3	136 ± 2	161 ± 2	174 ± 6	174 ± 8	185 ± 12	209 ± 5	208 ± 5
NaCl intake, ml./rat/day	54 ± 6	68 ± 6	75 ± 7	65 ± 6	70 ± 9	85 ± 6	86 ± 6	82 ± 5

Means ± standard errors.
* $P < 0.02$, †$P < 0.01$, §$P < 0.001$.

TABLE 2

Group	Organ weights ± S.E., mg./100 gm.			Mortality
	Adrenal	Kidney	Heart	
SC-5233	20 ± 2	740 ± 27*	274 ± 11†	3/12
Placebo	20 ± 2	843 ± 50	378 ± 22	3/12

* $P = 0.13$, †$P < 0.001$.

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SC-5233 acted by interfering competitively with the mineralocorticoids secreted by the regenerating adrenal. However, it is quite possible that the blood pressure effects of the compound are independent of its blocking effect on the renal excretory actions of mineralocorticoids. This was suggested by the observations that the 19-nor derivative of SC-5233, though a more potent blocker at the kidney level (2, 3), lacked the hypotensive action of SC-5233 in metacorticoid hypertension (5, 6), a disease that is initiated by corticoid-sodium overdosage but that soon becomes self-sustaining. With other renal blockers as well, including steroids with and without the lactone configuration, there has been a general lack of correlation between hypotensive action and the ability to block the electrolyte excretory patterns produced by mineralocoticoids. Therefore, it is not possible at this time to suggest a mechanism for the effect of SC-5233 on adrenal regeneration hypertension.

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Acknowledgment

The valuable technical assistance of Miss Esther Lacunza is gratefully acknowledged.

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LIPID DEPLETION OF THE ZONA GLOMERULOSA OF THE RAT ADRENAL CORTEX AFTER ADMINISTRATION OF 9-ALPHA-FLUOROHYDROCORTISONE[1]

ABSTRACT

Nine-alpha-fluorohydrocortisone is physiologically ambivalent, acting as a potent glucocorticoid and mineralocorticoid. Administration of 0.25 mg. of this material per 100 gm. of body weight, subcutaneously, 3 times weekly, for 7 weeks to young and old, female, Sprague-Dawley rats caused selective lipid depletion of the

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zona glomerulosa. This lipid-depleting effect of 9-alpha-fluorohydrocortisone was practically identical to changes induced in rats given desoxycorticosterone acetate. However, the younger rats required the "conditioning" effect of exogenous ACTH given with the 9-alpha-fluorohydrocortisone before the glomerulosa lipid-depleting effect could take place. These results are interpreted to mean that, in the rat, the mineralocorticoid activity of [illegible] re manifest on the zona glomerulosa of the adrei [illegible] effect. The results also suggest that ACTH may have some conditioning effect on the responsiveness of the secretory activity of the zona glomerulosa in the rat.

The zona glomerulosa of the adrenal cortex secretes mineralocorticoids, e.g., aldosterone, desoxycorticosterone (1). High levels of desoxycorticosterone-like steroids (2), changes in sodium levels (3) and hypertensive vascular disease (4) have been associated with changes in the width or lipid content of this zone. Changes in the zona fasciculata have been correlated with circulating levels of the glucocorticoids (5). The morphological and functional implications of these cortical zones have been reviewed by Greep and Deane (5). Aldosterone excretion is augmented in congestive heart failure (6), eclampsia (7), nephritis and nephrosis (8), ascites (9) and hypertension (10). For this reason it would be important to learn more concerning the specific functional roles of the zones of the adrenal cortex, what controls them, and how they respond to exogenous and endogenous stimuli.

A recently prepared synthetic analogue of hydrocortisone, 9-alpha-fluorohydrocortisone has been demonstrated to possess a most unusual type of activity, i.e., it is a potent glucocorticoid (11–13) and at the same time, equal to or more potent than aldosterone as a mineralocorticoid (14–16). While studying this compound we encountered some unusual effects on certain endocrine organs. The most striking effect was on the zona glomerulosa of the adrenal which is reported here.

MATERIALS AND METHODS

This work is taken from a larger study, to be reported later, in which 21-day-old and 12 month-old female, Sprague-Dawley rats were unilaterally nephrectomized, and treated with a variety of steroids. Regular rat chow (Rockland) was fed and no saline was added to the drinking water. Nine-alpha-fluorohydrocortisone[2] (in saline) was given as 0.25 mg./100 gm. of body weight (b.w.), subcutaneously (s.c.), three times weekly to both the young and old rats. Similarly, cortisone (2.5 mg./100 gm. b.w., s.c.) and desoxycorticosterone acetate (DCA) (2.5 mg./100 gm. b.w., s.c.) were given separately to animals on a similar regimen. Additional groups of young and old rats were given the above dose of 9-alpha-fluorohydrocortisone in conjunction with $\frac{1}{3}$ unit of ACTH (gel)/100 gm. of b.w., s.c., three times weekly. After 7 weeks of treatment the animals (12 animals per group) were sacrificed. Adrenal glands were fixed in 10% neutral formalin, sectioned on a freezing microtome at 10 μ, and stained for lipid content with Sudan orange (hematoxylin counterstain) and Sudan black B (Meyer's carmalum counterstain).

RESULTS

The very potent glucocorticoid effects of 9-alpha-fluorohydrocortisone were demonstrated by the severe involution of the thymus and spleen in both young and old animals. However, loss in body weight and adrenal atrophy were not marked. Mineralocorticoid effects were manifested by the development of severe hypertension, kidney damage, and increased heart size. Severe arteriosclerosis was found in the aortae of the older rats (to be reported). This may, or may not be a manifestation of mineralocorticoid activity. Concomitant with the above changes there was complete and selective depletion of the lipid material in the zona glomerulosa (Fig. 3) in all of the older animals receiving this hormone. The lipid depletion of the zona glomerulosa produced by 9-alpha-fluorohydrocortisone resembled similar depletion in rats given DCA. However, an interesting age factor appeared to be operative in the zona glomerulosa response to

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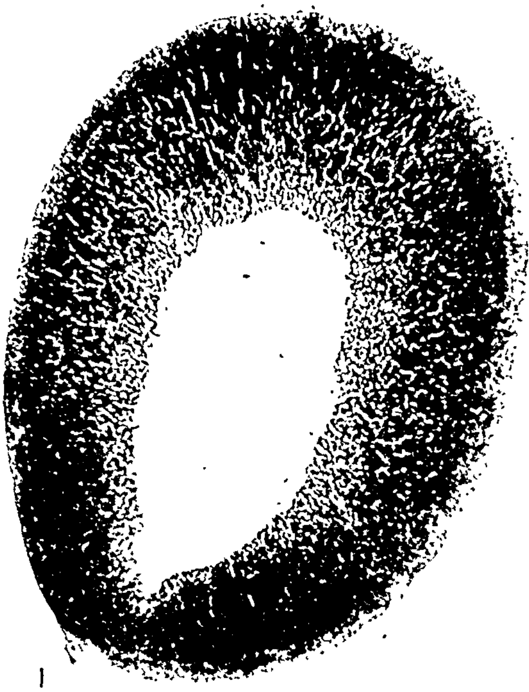
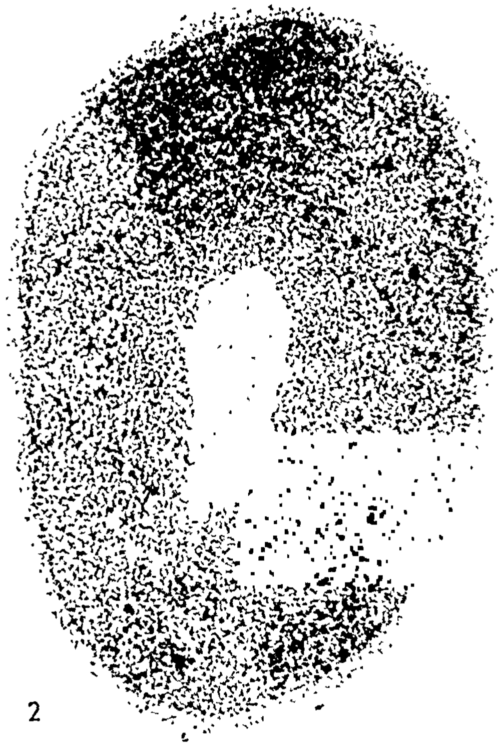
[2] We are indebted to Dr. Henry A. Strade of the E. R. Squibb Company for a gift of 9-alpha-fluorohydrocortisone.





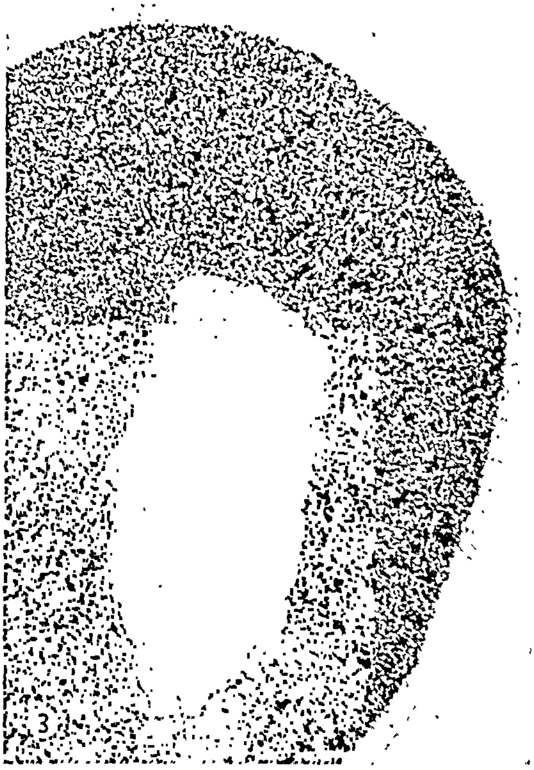


FIG. 1 Adrenal cortex of non-treated, young, female weanling, Sprague-Dawley rat. All zones are filled with lipid. (Sudan black B, 10 μ, ×50.)

FIG. 2. Complete, selective depletion of the zona glomerulosa of a young, female weanling given 0.25 mg. of 9-alpha-fluorohydrocortisone and $\frac{1}{3}$ unit of ACTH (gel) per 100 gm. body weight, subcutaneously, once daily, every other day, for 7 weeks. (Sudan black B, 10 μ, ×50.)

FIG. 3. Lipid depletion of the zona glomerulosa of a year-old, female Sprague-Dawley rat given 9-alpha-fluorohydrocortisone only (same dose as in Fig. 2). Note greater hypertrophy of cortex in older animal. (Sudan black B, 10 μ, ×50.)

9-alpha-fluorohydrocortisone. All animals, young and old, given DCA showed lipid depletion of the zona glomerulosa. None of the younger animals given 9-alpha-fluorohydrocortisone alone exhibited any evidence of lipid depletion of the zona glomerulosa. If the younger animals were given ACTH in conjunction with the 9-alpha-fluorohydrocortisone they consistently showed very marked lipid depletion of the zona glomerulosa comparable to that seen in the older animals (Fig. 2). In no case (total of 264 animals) was this selective lipid-depleting effect on the zona glomerulosa observed except in those cases mentioned above.

DISCUSSION

The disappearance of lipid from one of the zones of the adrenal cortex usually connotes decreased biosynthesis by that zone. In these experiments DCA and 9-alpha-fluorohydrocortisone both caused selective depletion of the zona glomerulosa. Cortisone produced disuse atrophy and lipid depletion of the zona fasciculata. It is particularly interesting that 9-alpha-fluorohydrocortisone which is "ambivalent" physiologically (11–16) showed no histological effects on the zona fasciculata but very dynamic lipid-depleting effect on the zona glomerulosa; this, in spite of the fact that it had marked glucocorticoid activity in these same animals, i.e., severe involution of the thymus and spleen. This selective histophysiological effect by 9-alpha-fluorohydrocortisone on the zona glomerulosa is more in keeping with clinical experience which has shown it to be equal to or more potent than aldosterone (14–16). These histological results conflict with certain physiological studies which demonstrated that 9-alpha-fluorohydrocortisone shows little sodium-retaining activity in the rat but produces marked sodium retention in other species such as dog and man (13).

It is interesting that 9-alpha-fluorohydrocortisone did not cause lipid depletion when given alone to the younger rats. However, the fact that it did cause lipid depletion of this zone when given in conjunction with ACTH is provocative. In 1955, Wexler and Rinfret (17), reported that, "certain, but not all species of ACTH will specifically deplete the lipid in the zona glomerulosa of the rat." On the basis of these histological observations they suggested that aldosterone and aldosterone-like steroids may be released under the influence of ACTH. Venning et al., (18) have recently shown that *in vitro* preparations of rat adrenal do produce increased amounts of aldosterone in response to certain ACTH preparations. It might well be that the older rats responded to the mineralocorticoid effects of 9-alpha-fluorohydrocortisone because of higher circulating levels of ACTH which "conditioned" the zona glomerulosa to the mineralocorticoid. On the other hand, younger rats having a lower titre of circulating ACTH could not respond to the 9-alpha-fluorohydrocortisone until exogenous ACTH was introduced. The well known pituitary ACTH-suppressing effects of 9-alpha-fluorohydrocortisone (19–21) may also have been operative.

Acknowledgment

The author is indebted to Dr. Benjamin F. Miller for encouragement and many helpful suggestions. He would also like to thank Miss Janice Doron, Senior Histology Technician for the preparation of histological specimens, and Miss Jane Hutzelman, medical photographer, for the fine photomicrographs.

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A MODIFICATION OF THE BATES-CORNFIELD BIO-ASSAY FOR THYROTROPIN[1,2]

ABSTRACT

Day-old chicks with low thyroidal uptake of radioiodine were used in the Bates-Cornfield bio-assay for thyrotropin. This was made possible by introducing an easily available correction factor for the circulating extrathyroidal radioactivity. It was concluded that the dose-response curves obtained by this method compare favorably to those obtained by the original method.

The method of Bates and Cornfield (1) is one of the few current methods that may be useful in the determination of thyrotropin (TSH) in human blood. This method is dependent on the use of chicks with a high uptake of I^{131}, and they are not easily available. A modification has therefore been worked out that permits the use of chicks with a low uptake of radioiodine.

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[1] Abridgment of portion of thesis submitted by Dr. Frey to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

[2] The radioisotopes used in the studies reported on in this paper were supplied by the United States Atomic Energy Commission.

MATERIAL AND METHODS

Plymouth White Rock chicks of mixed sexes were used. They were received on the day of hatching and were given I^{131} on arrival. They were kept at 35° C in brooders housing 50 chicks; they had free access to tap water but were starved routinely.

Solutions of propylthiouracil (PTU) and thyroxine were prepared as described by Bates and Cornfield (1). The daily dose per chick was 8 gamma thyroxine and 0.5 mg. propylthiouracil. The thyrotropin used was Armour's Lyophilized Thyrotropin, lot No. R 491075. Serial dilutions of thyrotropin were prepared immediately before use in 0.1% ovalbumin. The radioiodine used was carrier-free, pyrogen-free sodium iodide^{131}. The dose administered (1 to 5 μc.) was too small to cause any significant radiation damage to the thyroids of the chicks (Skanse (2)), and the birds used in the experiments have subsequently grown to be normal adults. The radioiodine was injected subcutaneously in the nape of the neck in a volume of 0.2 cc. Whole body counts immediately after injection on several occasions showed that the whole dose was retained. Injections of solutions of thyrotropin and of thyroxine also were done in the same way and at the same site.

A well-type scintillation crystal, connected to a scaler, was used for determination of radioactivity. Best results were obtained by using a lead collimator ½ inch in thickness with a hole in the center 1 inch in diameter. The lead collimator was covered by a thin aluminum plate with a center mark. The chick was held in one hand, breast down, with the sternal notch above the center mark. Firm pressure maintained a constant distance to the crystal. The bird usually remained quiet during the procedure. The reproducibility of this counting method was ±7%. On counting over the tail, the chick was pushed headwards as far as possible with the 1-inch hole still covered by the tail.

RESULTS

Figure 1 shows the effect of introducing the "tail count" as a correction factor for the extrathyroidal radioactivity in the neck (the "body background"). The difference between the count obtained over the neck and the count obtained over the tail may be taken to represent the true thyroidal radioactivity, that is the "thyroid count." The relative constancy of the thyroid count will enable one to use this count as a baseline which is largely uninfluenced by changes in extrathyroidal radioactivity.

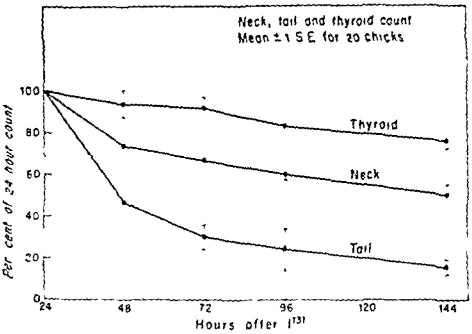

FIG. 1. Neck, tail and thyroid counts at various intervals after injection of I^{131}.

In Figure 1 the neck, the tail and the thyroid counts for each chick are expressed as percentages of the count for each category obtained 24 hours after the injection of I^{131}. Each point on the curve represents the mean value for 20 chicks. Propylthiouracil and thyroxine were administered in the usual doses every 24 hours, starting 24 hours after the injection of I^{131}.

It is apparent from Figure 1 that the curve for the thyroid count shows a slower fall than the curve for the neck and the tail counts. The 24% loss from the thyroid over a 5-day period conforms well with the loss of radioactivity from the blocked thyroid as determined by removal and direct counting of the glands (loss of 5% per day).

In trying to estimate how representative the tail count is for the extrathyroidal count in the neck, day-old chicks were given 0.5 mg. of propylthiouracil and 8 μg. of thyroxine 12 hours and again 1 hour before the injection of I^{131}. The effectiveness of propylthiouracil and thyroxine in preventing uptake of radioiodine by the thyroid was demonstrated by

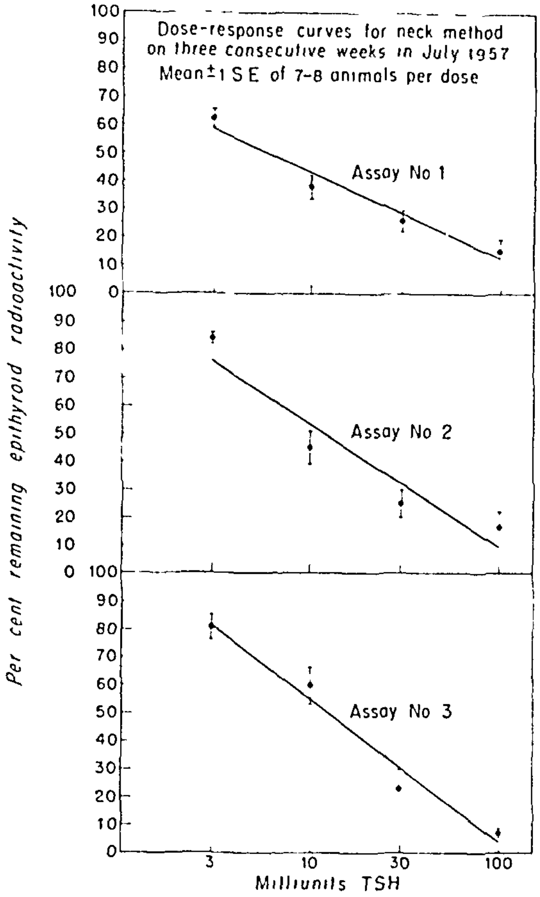

FIG. 2. Three dose-response curves for thyrotropin.

killing some of the birds 24 hours after the injection of I^{131} and determining the uptake of I^{131} by the thyroids. The glands showed a negligible uptake of I^{131}. Counts were made over the neck and the tail 3, 6, 12, 24, 48, 72 and 92 hours after the injection of I^{131}. The counts showed a parallel fall with a ratio of tail count to neck count varying slightly around 0.80. This indicated that the tail count at all times represented an 80% correction for the extrathyroidal radioactivity in the neck.[3]

The influence of the backscatter from the thyroid on the tail count was found negligible, as proved by experiments using varying doses of I^{131} applied externally with adhesive tape over the thyroid.

The tail count was found to be an easily available and fairly accurate correction factor for the body background, and assays were carried out by means of the technic of Bates and Cornfield (1). The chicks arrived in the evening and were given I^{131} on arrival; the first reading was obtained in the morning 36 hours later. This reading served as the 100% value for each chick. The chicks were not selected according to body weight or thyroid uptake but they were picked at random with 7 to 8 chicks per dose. After the initial reading, thyrotropin was given in divided doses, one injection daily for 3 days. On the fourth day only a count was done; it was expressed in per cent of the initial reading after correction for physical decay of the isotope. Figure 2 shows the dose-response curves obtained on three consecutive weeks in July, 1957. Each point represents the mean thyroid count for each dose level. Figure 2 gives the regression lines, slope and index of accuracy for the three assays.

COMMENT

The three curves differ slightly in their characteristics. This is partly artificial, because the curves reach an asymptote before the dose of 100 milliunits (U.S.P.) of thyrotropin. Therefore, when the dose of 100 milliunits is included in the computation of the regression line, the slope will be artificially lowered. This factor is especially prominent in assay No. 1. For the three assays, the slopes were −30, −44 and −51; the λ values were 0.36, 0.26 and 0.20, respectively.

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[3] As was pointed out in the text, the tail count represents the general level of I^{131} in the tissue. However, a few chicks have a disproportionately high tail count on the initial reading, probably due to accumulation of radioactive material in the excretory organs. These animals were excluded for assay purposes if the ratio of neck count to tail count on the initial reading was less than 3:1. About 10% of the animals do not meet this requirement.

Only minor variations in animal sensitivity were encountered from week to week in July. However, at least a tenfold variation in animal responsiveness occurs. Thus in the late fall months the extinction-point was higher than 10 milliunits, while in February, 1958, it was 0.3 milliunit. The curves presented compare favorably with the original method of Bates and Cornfield.[4] Sensitivity, accuracy and range are almost identical. The slope may be higher in the original method, but, considering the artificially lowered slope in the curves presented here, this difference is not so great as to be of practical importance.

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[4] The original method of Bates and Cornfield requires chicks with an average thyroid uptake of radioiodine of at least 20 per cent of the dose. The chicks used in the present study had an average 24-hour uptake from May to October of 8%. It is obvious that in chicks with a low uptake the extrathyroidal radioactivity in the neck will influence the neck count to a disturbing degree. But this also may hold true for chicks with a high uptake. Thus we did not find any correlation between the radioactivity in the thyroid and the radioactivity in the rest of the chick (called the "carcass") when counts were made separately 24 hours after the injection of I^{131}. Even chicks with a relatively high thyroidal uptake (in excess of 20%) might have retained more than 40% of the dose in the carcass. Therefore, the extrathyroidal radioactivity may have to be taken into consideration even in a chick with high thyroidal uptake.

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AN EFFECT OF VASOPRESSIN ON RENAL WEIGHT

ABSTRACT

It has been found that the administration of "Pitressin" or synthetic lysine vasopressin will bring about an increase of the weight of kidneys of hypophysectomized rats. An effect was noted with as little as 10 milliunits of synthetic lysine vasopressin. Normal rats and operated control or saline injected hypophysectomized rats did not respond with a change in renal weight.

Male Sprague-Dawley rats about 6 months in age were used in the study. The hypophysectomized rats weighed 100–150 gm. and were three months postoperative with no observed growth response. The hormone preparations were "Pitressin" (Parke, Davis) and synthetic lysine vasopressin (LVS_2) supplied by Dr. Vincent du Vigneaud. The animals were anesthetized with ether (identical results were obtained with chloralose), one kidney was removed and the hormone preparation was administered in the tail vein in 0.1–0.3 ml. saline. After one hour, the remaining kidney was removed, the organs were freed of connective tissue, blotted on a filter paper and weighed.

RESULTS AND DISCUSSION

The increases of kidney weight versus dose of administered hormone are shown in Figure 1; six animals were used for each of three of the points. The average deviation

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was less than 4%. At the dosage of 60 milliunits (the third point) 16 hypophysectomized rats were tested. At this level the average increase was 13.3% and the average deviation from this average was ±4.6%. In a group of 9 normal rats of the same strain and age receiving 600 milliunits of vasopressin the increase was 0.3±3.6%; in a group of 6 saline-injected hypophysectomized rats, the change of renal weight was −1.2±2.8%. At higher levels of vasopression (2000 and 3000 milliunits) the increase in renal weight at one hour approximated 20%; it was not possible to distinguish between these two levels. In the lower dosage levels, the difference between 6 and 12 milliunits was not easily distinguishable with the small samples. In all 35 control animals, the average change in renal weight was 3.2%.

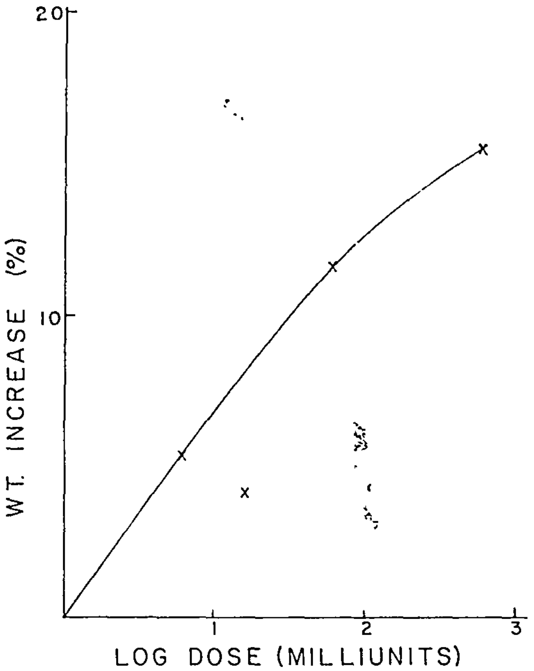

Fig. 1. Increase in kidney weight plotted against log dose (in milliunits) of synthetic lysine vasopressin-LVS2.

Some studies were made of the maximally stimulated kidneys (2 units vasopressin); the kidneys were homogenized in water and the content of hemoglobin (as oxyhemoglobin) and of protein (1) was determined. There were no differences noted in the analyses of 6 pairs of kidneys and one must conclude that water was the principal component responsible for the gain in weight. The effect would seem to be quite specific; a variety of proteins and protein hydrolysates injected in amounts of 0.1 to 1.0 mg. had no effect on renal weight. It would appear that the effect is sensitive and specific enough to serve as a test for moderate amounts of vasopressin. The time relationship has not been investigated in detail but easily detectable differences were noted with 60 milliunits at 15 minutes and the effects at 30 and 60 minutes were nearly identical.

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[1] We are indebted to Frances Jobson for technical assistance.

OXYTOCIN AND OXYTOCIC SUBSTANCE IN BLOOD EXTRACTS BEFORE, AND FOLLOWING, COPULATION IN SHEEP

ABSTRACT

Ewes in natural oestrus showed no increase in the concentration of oxytocin in blood extracts following stimulation by copulation.

There is indirect evidence that, during natural and artificial insemination, oxytocin is released into the blood from the hypothalamic-posterior-pituitary complex (1). Milk ejection in lactating mothers has been frequently observed in association with coitus, and it has been postulated in animals that the powerful uterine contractions induced by the stimulus of copulation may assist in the rapid transit of spermatozoa into the fallopian tubes.

To our knowledge no one has actually measured oxytocin in the blood in these circumstances, so it was decided to investigate the effect of copulation on the blood level of oxytocin in sheep.

METHOD

Samples of jugular blood (approx. 10 ml.) were taken from 7 ewes (in natural oestrus) before, and immediately after, copulation. The blood specimens were then extracted and assayed for oxytocic activity, oxytocin and oxytocic substance as previously described (2).

RESULTS AND DISCUSSION

Table 1 shows that in 7 ewes the total oxytocic activity of the blood extracts decreased in 6 and increased in 1. The changes observed in ewes 6 and 7 were of doubtful significance, and if these are exluded then a fall in total oxytocic activity was registered in all cases. In 5 ewes from whom the blood extracts were assayed for oxytocin and oxytocic substance, the level of oxytocin was unchanged in 4 and increased in 1, whilst the concentration of oxytocic substance did not significantly increase in any.

TABLE 1. OXYTOCIC ACTIVITY OF ACID-ALCOHOL EXTRACTS OF BLOOD (SHEEP) BEFORE, AND AFTER, COPULATION

No.	Oxytocin	Tot. activity	Oxytocic substance	Tot. activity	Percentage change in total activity	Percentage change in oxytocin	Percentage change in oxytocic substance	Oxytocin		Oxytocic substance	
	Percentage		Percentage					Mu /ml blood			
	Before	After	Before	After				Before	After	Before	After
1					−88 9	—	—	2 24		1 2	
2					−32 6	—	—	—		—	
3	0	0	100	100	−35 3	0	−11 8	0	0	0 17	0 15
4	0	0	100	100	−20 0	0	−20 0	0	0	0 25	0 20
5	0	0	100	100	−16 6	0	+10 3	0	0	0 29	0 32
6	0	0	100	100	+11 1	0	0	0	0	0 45	0 45
7	1 25	20 6	98 75	79 4	− 7 5	1500	−26 6	0 01	0 16	0 79	0 58

It is interesting to note that, in these experiments on sheep, there is virtually no oxytocin detectable in the blood extracts. In this respect sheep differ considerably from rats (3) since in the latter the concentration of oxytocin is invariably in the order of several mu./ml. blood. In sheep the oxytocic activity of blood is almost entirely due to the presence in the extracts of oxytocic substance. It is now thought that the latter is a polypeptide, and that it is not to be confused with such other oxytocic factors as oxytocin, potassium, acetylcholine, 5-hydroxytryptamine, hypertensin, vasopressin, pepsitensin, pepsitocin, pepsanurin, substance P, pain-producing substance or kinin (bradykinin, kallidin, substance U or substance Z).

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COMPARISON OF *IN VITRO* AND *IN VIVO* ASSAYING PROCEDURES FOR RAT ADENOHYPOPHYSIAL CORTICOTROPHIN[1]

ABSTRACT

The same rat pituitary extract was concurrently assayed against the same standard (U.S.P. Reference Standard Corticotropin) by procedures respectively based on *in vitro* corticoidogenesis, and on *in vivo* corticoidogenesis and adrenal ascorbic acid depletion. Nearly identical values (20–24 mu. ACTH/mg. fresh tissue) were obtained on the basis of *in vivo* corticoidogenesis and ascorbic acid depletion, whereas a markedly higher concentration (62 mu./mg.) was recorded on the basis of *in vitro* corticoidogenesis. These findings do not support the hypothesis of Birmingham *et al.* of a higher *steroidogenic/ascorbic acid-depleting* activity ratio in rat pituitary extract than in the reference standard. The discrepancy in the results yielded by the two types of procedures appears to be related to differences in the nature (*in vivo* or *in vitro*) rather than in the end-points (adrenal ascorbic acid depletion or stimulation or corticoidogenesis) of the assays.

Consistently higher values for pituitary corticotrophin concentration in the intact rat have been obtained with Saffran and Schally's *in vitro* corticoidogenic assay (1) than with Sayers' *in vivo* adrenal ascorbic acid depletion method (2). Due allowances being made for the different strains of rats and extraction procedures used by various groups, the values recorded, in terms of milliunits (mu.) of ACTH per mg. of fresh tissue, range from 10 to 38 (3–6) with Sayers', and from 52 to 241 (7, 8) with Saffran and Schally's assay. A mean concentration of 64.6 ± 1.8 mu./mg. was recorded by one of us (9) from one hundred *in vitro* assays (average λ:0.106 ± 0.004) of 0.1N HCl-extracted pituitaries from intact male Sprague-Dawley rats.[2]

To account for the discrepancy in the results yielded by the two assaying procedures, Birmingham *et al.* (7) suggested that the *steroidogenic/ascorbic acid-depleting* (S/AA) activity-ratio might be higher in native (pituitary extract) than in more purified (Reference Standard) corticotrophin. The following experiment was aimed at testing this possibility by concurrently assaying the same rat pituitary extract against the same standards, through procedures respectively based on *in vitro* corticoidogenesis, and on *in vivo* corticoidogenesis and adrenal ascorbic acid depletion.

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[1] This investigation was supported by research grants PHS B-959 and PHS A-1276 from the Institute of Neurological Diseases and Blindness and the Institute of Arthritis and Metabolic Diseases, the National Institutes of Health, Public Health Service.

[2] Obtained from Holtzman Rat Company.

One day prior to assay, the adenohypophyses from two adult male Sprague-Dawley rats, killed by decapitation, were quickly removed from the skull, separated from the neural lobe, wiped clean of blood, weighed to the nearest 0.05 mg., pooled in a 15 ml. conical centrifuge tube containing 0.4 ml. of 0.1N HCl, and ground with a glass rod and a few grains of sand. The rod was rinsed into the tube with an identical volume of 0.1N HCl, and the stoppered extract, stored in the deepfreeze. A weighed sample of U.S.P. Reference Standard Corticotropin dissolved in 0.1N HCl was stored at the same time. On following morning, extract and standard were thawed, adjusted to 0.01 normality, and left for two hours at room temperature. A four-point-two-dose ($D^1/D^2 = \frac{1}{3}$) design was used for *in vitro* and *in vivo* assays. On the basis of previous experiments, the S^2 solutions of the standard were respectively adjusted to 100 mu. ACTH/ml. for the *in vitro*, and to 5 mu./ml. for the *in vivo* assays; the U^2 solutions of the extract, to 1.9 mg. pituitary/ml. for the *in vitro*, and to 0.22 mg./ml. for the *in vivo* assays. Aliquots of 0.3 ml./100 mg. adrenal were used in the *in vitro*, of 0.3 ml./100 gm. B.W., in the *in vivo* assays.

The *in vitro* assays were performed by the technique of Saffran and Schally (1). The *in vivo* assays were based on the a) adrenal ascorbic acid, b) adrenal corticosteroid, and c) plasma free-corticosteroid responses of 24-hour hypophysectomized male rats (Hormone Assay Laboratories, Chicago; 6 rats per dose) to the injection of extract or standard. The solutions were injected in a randomized fashion, by the intrajugular route, under ether anesthesia. 15 minutes later,[3] blood was collected with a heparinized syringe from the abdominal aorta for determination of the plasma free corticosteroids by the fluorometric method of Silber, as modified by Guillemin *et al.* (10), and both adrenals were removed, dissected free of fat, and weighed to the nearest 0.1 mg. The left gland was extracted in 4% trichloracetic acid for ascorbic acid determination by a modification (solutions incubated for 15 minutes (vs. 180) at 100° C (vs. 37° C) of Roe and Kuether's method (11). The right gland was extracted in alcoholic saline prior to corticosteroid determination by fluorometry (12). The results, computed and tested for validity according to Bliss' methods for factorial analysis and analysis of variance (13), were expressed in mu. of ACTH per mg. of fresh tissue, with confidence limits (antilog $C^2M \pm CtSm$) for odds of 19 in 20. Equal emphasis was placed on the internal (*within* assays) and external (*between* assays) variances of the combined estimates (unweighted logarithmic means) by computing their standard errors from the averages of the crude and unweighted $S\bar{m}$'s (13).

TABLE 1. COMPARATIVE ASSAYS OF THE SAME RAT PITUITARY EXTRACT AGAINST THE SAME STANDARD BY *in vitro* AND *in vivo* TECHNIQUES

Type of assay	ACTH per mg. fresh tissue (mu.)	95% confidence limits (mu.)	Index of precision (λ)
In Vitro Corticoidogenesis			
#1	69.5	45.8–116.6	0.080
#2	42.5	23.5– 69.2	0.104
#3	60.8	46.8– 80.3	0.057
#4	80.6	40.2–314.7	0.157
Mean ±S.E.	61.7 ±7.2		0.099 ±0.026
In Vivo			
A. Adrenal Ascorbic Acid	20.8	15.8– 27.1	0.081
B. Adrenal Corticosteroids	20.1	18.3– 22.2	0.028
C. Plasma Free Corticosteroids	24.4	20.4– 27.1	0.034
Mean ±S.E.	21.7 ±1.2		0.048 ±0.017

RESULTS AND CONCLUSION

Nearly identical values were obtained on the basis of *in vivo* corticoidogenesis and ascorbic acid depletion, whereas a markedly higher concentration was again recorded on the basis of *in vitro* corticoidogenesis (Table 1). These findings do not support Birmingham *et al.'s* hypothesis of a higher S/AA activity ratio in rat pituitary extract than in the reference standard. The discrepancy would thus appear to be related to differences in the nature (*in vivo* or *in vitro*) rather than in the end-points (adrenal ascorbic acid depletion or stimulation of corticoidogenesis) of the assays. Further studies aimed at elucidating this problem are reported elsewhere (9).

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[3] This interval was observed to correspond to maximal adrenal ascorbic acid and corticosteroid responses to ACTH (10).

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[4] Dalland Fellow of the American Philosophical Society.

MODIFICATION OF THE CATABOLIC ACTION OF ACTH IN RATS FED A DIET HIGH IN POTASSIUM[1]

ABSTRACT

Groups of normal female rats were maintained on a constant food intake and daily urine collections were made for nitrogen analysis. Experimental animals received an extra 600 mg. per day of KCl in their diet. ACTH assaying 15 USP units per mg. was given in doses ranging from 10 μg. to 200 μg. per rat per day. At doses of ACTH below 100 μg. per rat per day no significant increase in urinary nitrogen occurred in those animals on the high KCl diet whereas it did occur in animals on the control diet. With doses of ACTH of 100 μg. or above per rat per day it occurred in both groups, but was more marked in the control animals.

In a previous paper from this laboratory (1) it was reported that the inclusion daily of an extra 600 mg. of KCl in the diet of normal female rats inhibited the catabolic effect of administered ACTH. In these experiments ACTH administration was started

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at such low dosage that no catabolic effect was observed either in the control animals or in the animals on the high KCl intake. As the dose of ACTH was increased, a definite catabolic effect appeared in the control animals. At this time no group of animals on a high KCl intake showed a statistically significant increase in nitrogen excretion, although some groups of animals on the high KCl intake did show small increases in urinary nitrogen. Subsequently, Rupp, Paschkis, and Cantarow (2), using tube fed animals receiving amounts of extra KCl identical to those employed in the experiments reported by this laboratory were unable to demonstrate an inhibition of the catabolic effect of either ACTH or cortisone. Their experiments differed, however, from those reported from this laboratory in that they used only one dose level, and a fairly large one, of both ACTH and cortisone. The dose of cortisone which they employed was 5 mg. per rat per day and they used 10 units of ACTH per rat per day. We have carried out experiments in an effort to repeat the original observation of Whitney, Bennett, and Li (1). Also, these experiments have been designed to determine whether if one used progessively larger doses of ACTH, its catabolic effect would reappear even though a diet high in KCl were being fed.

METHOD

The animals used, the diet, the methods of collection of urine and of analyses, the control of food intake and the amount of extra KCl added to the diet was identical with those previously reported (1). There were five animals in each group of animals in each experiment. Urine collections were started after a period of a week or more in the metabolism cages. The ACTH used was the ACTH preparation E obtained by elution from oxycellulose (3). The preparation assayed 15 USP units per mg. and was administered subcutaneously in bees wax.

RESULTS AND COMMENTS

The results of the experiments in which ACTH was administered are reported in Table 1. It will be noted that in three different experiments in which 40 μg. of ACTH was given per day a significant increase in urinary nitrogen occurred in the groups on the control diet on two occasions, whereas no significant increase occurred in any group on the high KCl diet. When the dose of ACTH was doubled to 80 μg. per day a significant increase in urinary nitrogen occurred in one of the two control groups but in neither of the groups on the high KCl diet. In four experiments when the dose of ACTH was 100 μg. or more per day significant increases in urinary nitrogen occurred in all control groups and in one of the groups on the high KCl intake. Also it is apparent from the table that in seven of nine periods there was an increase in urinary nitrogen when ACTH was given to animals on the high KCl diet. However, in each of these instances the increase in urinary nitrogen of the animals on the control diet was greater than that

TABLE 1. CHANGE IN URINARY NITROGEN OF ACTH TREATED ANIMALS

Experiment		[illegible] mg./day	Average change in urinary nitrogen [illegible] /day		[illegible] /day		[illegible] /day		[illegible] /day	p	[illegible] /day	p
15D	Control	402±3.7	+37	.03								
	High KCl	400±2.6	+13	.10								
15E	Control	428±7.3	− 4	>.80	+21	.10			+29	.05		
	High KCl	435±4.7	−12	>.80	−17	>.80			− 9	>.90		
15B	Control	323±4.6	+40	<.01	+47	<.01			+67	<.01		
	High KCl	344±7.1	+ 6	.60	+12	.50			+24	.10		
15A	Control	348±4.9									+55	< 001
	High KCl	336±4.9									+26	10
15F	Control	297±4.5					+110	<.001				
	High KCl	291±4.9					+ 47	03				

* The control period was six or seven days in duration.
† The treatment period was three or four days in duration.

of the animals on the high KCl diet. With increasing doses of ACTH its catabolic effect is more marked both in the control animals and in those on the high KCl intake. Thus, the average change in urinary nitrogen of control animals receiving less than 100 μg. of ACTH per day was an increase of 28 mg. per rat per day; whereas, the average change in high KCl animals at the same dosage was less than 1 mg. per rat per day. At doses of 100 μg. or more per day of ACTH the average increases in urinary nitrogen were 65 mg. per rat per day for those on the control diet and 28 mg. per rat per day for those on the high KCl diet.

In confirmation of previous results (1) there was no demonstrable difference in adrenal size when the weights of the adrenals of the ACTH treated animals on the control diet and the adrenals of the ACTH treated animals on the high KCl intake were compared. Likewise, when these adrenals were studied microscopically after staining by the Flexner Grollman technique, (4) no differences between the two groups were found.

It is worthy of note that in these experiments at daily doses of ACTH below 1.5 units per rat per day, no significant increases in urinary nitrogen occurred in animals on the high potassium diet. At doses of 1.5 units or more per rat per day significant increases in urinary nitrogen occurred in both groups. As pointed out earlier, Rupp, Paschkis, and Cantarow used only doses larger than these in their experiments in which they reported failure to confirm the results of Whitney and Bennett.

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A TECHNIQUE FOR REMOVING THYROID GLANDS FROM ANURAN LARVAE[1]

ABSTRACT

It is pointed out that removal of thyroidal primordia from embryos is unsatisfactory as a means of obtaining thyroidectomized larvae since the gland often regenerates. A technique for removing the gland from the tadpole is described and cited as producing uniformily good results.

Thyroidectomized tadpoles are occasionally needed in large numbers for endocrinological investigations. Allen (1) has described a technique for removing thyroid anlagen from embryos at tailbud stages of development, but his technique is rather tedious and time consuming. Also, Dr. William Etkin (personal communication) reports that he, as well as other workers, find that the gland regenerates in at least 50% of animals operated upon in the tailbud stage even when the entire floor of the pharynx is removed. The technique to be described was cited earlier (2). It has been used in the successful extirpa-

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tion of thyroid glands from *Rana pipiens*, *R. palustris*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor* at all stages of larval development; but the operation was most conveniently carried out on animals at the stage during which yolk consumption is being terminated and feeding is beginning to take place. At that stage, the thyroid still contains yolk granules and is easily distinguished from surrounding tissues so that complete removal of the gland is readily accomplished. Furthermore, younger larvae seem to be less adversely affected by operational trauma than older animals.

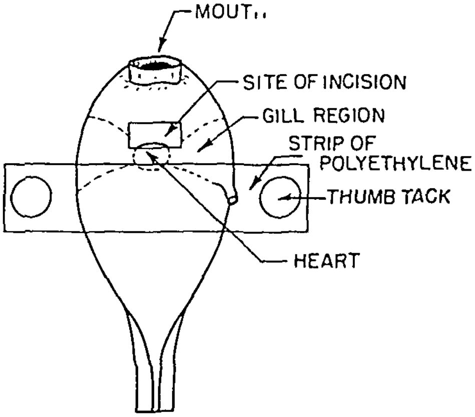

Fig. 1

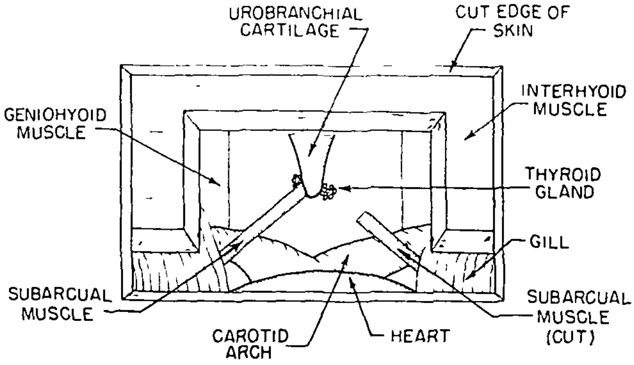

Fig. 2

In preparation for the operation, the tadpole is anesthetized in tricaine methanesulfonate (MS-222, M. Sandoz, New York) at a concentration of 1:3000 in spring water. The animal is then placed on its back within a depression molded in the bottom of a wax-lined operating dish. It is secured by means of a strip of flexible plastic material (polyethylene sheeting) which, in turn, is held in place with thumbtacks (Fig. 1). Cutting is done under a dissecting microscope by inserting a knife edge ground on the tip of a dissecting needle beneath the tissue to be cut and then working the tips of a pair of watchmaker's forceps against the knife edge through the tissue. First, a rectangular piece of skin is removed just anterior to the pericardial cavity, exposing the medial portion of the interhyoid muscle. The interhyoid is a relatively broad, flat muscle with fibers running transversely (Fig. 2). The posterior half of this medial portion (or the complete medial portion in younger animals) of the interhyoid is then removed. Through the opening thus created the urobranchial cartilage is seen extending ventrocaudad from the hypobranchial plate of the hyoid apparatus. A pair of subarcual muscles are attached to the urobranchial cartilage and run obliquely caudolaterad from it. When these subarcual muscles are transected, the lobes of the thyroid are seen lying against the hypobranchial plate lateral to the urobranchial cartilage, medial to the geniohyoid muscles, and craniodorsad to the carotid arches. Finally, the exposed thyroid lobes are removed by aspiration through a capillary pipette with a mouth-controlled suction tube attached to it.

Tadpoles operated on in this fashion required no special postoperative care. They were merely put in ordinary culture dishes or aquaria, where rapid healing ensued. Removal of the thyroid was demonstrated to be complete, and mortality was negligible.

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Acknowledgment

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[2] Operated by Union Carbide Corporation for the United States Atomic Energy Commission. (Work done under subcontract No. 701)

THE USE OF THE TOAD FOR BIOASSAY OF MAMMALIAN ANTIDIURETIC HORMONE (VASOPRESSIN)[1]

ABSTRACT

Injection of mammalian antidiuretic hormone (ADH) into toads produced increased water uptake and decreased urine production, resulting in weight gain over the pre-injection period. A bioassay for ADH, based on this response, has been developed for *Bufo marinus*, a large toad available in the United States. This method is an adaptation and modification of an assay devised by Buchborn for the European toad *Bufo* bufo. Details of the assay procedure are given, as well as instructions for maintaining the toads in the laboratory.

The role of the neurohypophyseal hormone, vasopressin, in body water and electrolyte balance has long been of interest to both physiologists and clinicians. The determination of circulating levels of this hormone has, however, been hampered by the capricious nature and limited sensitivity of the assay methods which have been available. Most of these methods depend on the excretion of an administered water load by rats or other mammals. But pain, trauma, stress, anesthesia, and even noise, temperature changes or strange environment may interrupt the expected diuresis in a water loaded animal, presumably by effecting an increase in endogenous hormone production.

Brunn (1) first pointed out that amphibians respond to mammalian neurohypophyseal extracts by increased water uptake through the skin. Many subsequent workers

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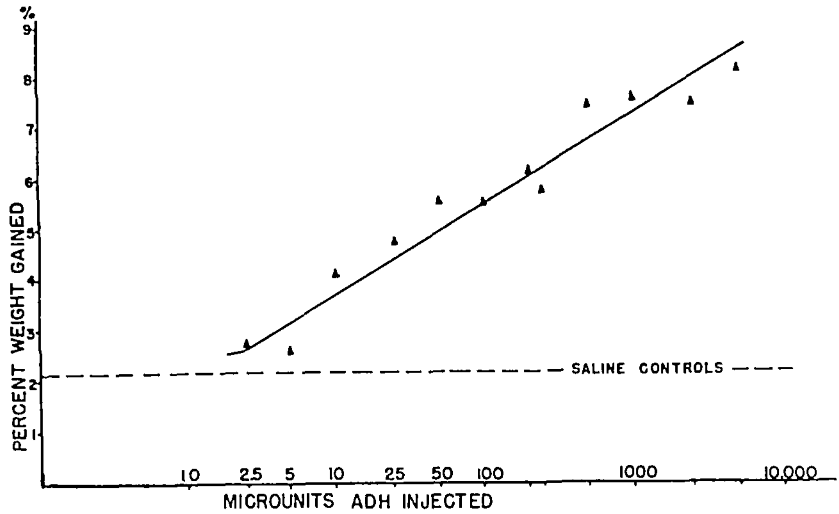

FIG. 1. Dose-response curve for *Bufo marinus* given various dosages of Pitressin[2] (3). The abscissa is a logarithmic scale, one microunit being one millionth of an International (pressor) Unit. Each triangular point represents the mean response of five toads at the third hour after injection. The dashed line represents the average weight gain, after the same interval, of toads injected with saline only. (The zero point on the ordinate scale represents the mean hydrated weight, or pre-injection weight, of each toad; since the injected fluid constitutes five percent of the animal's body weight, every animal will be heavier at the next weighing. However, this net gain is gradually lost by animals who receive only saline: it is maintained or increased under the influence of ADH).

have confirmed this fact, and have further found that toads are more sensitive than frogs to vasopressin. Buchborn (2) utilized this sensitivity in developing an assay method for antidiuretic hormone (ADH) using the European sand toad *Bufo bufo*. This author claims his method to be specific and sensitive from 10 μu. to 1500 μu. ADH.

We have adapted and modified this assay procedure for use with toads available in this country. The giant toad, *Bufo marinus*, has been used in the present study. This species is widespread in Central and South America, and its range extends northward into the southern tip of Texas. The animals are easily obtained because several supply houses stock them for pregnancy tests.

METHOD

For each assay, five male toads are hydrated by allowing them to remain in water overnight. The mean hydrated weight of each toad is then determined from three hourly weight determinations. After each weighing, the animal is caused to excrete his accumulated urine by catheterization with a blunt pipette, combined with slight pressure on the lower abdomen. Thus each weight determination represents base weight plus water taken up during the previous hour. Premature micturation with large variations in weight has not proved a problem. Each animal is then given an injection—5 cc. for every 100 gm. body weight—of vasopressin in saline,[2] into the dorsal lymph sac. Within two to five hours, depending on the size of the toad, a maximum weight gain (ADH) or return to original pre-injection weight (saline controls) is observed. For *B. marinus* weighing 80–120 gm., the mean response of five toads at three hours after injection is taken as the end point.

The magnitude of the response is directly proportional to the dosage of hormone injected (Fig. 1). When response of the toads (per cent weight gain) is plotted against log-dose, the curve follows a straight course from less than 5 μu. to 5,000 μu. (5 mu.).

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[2] Pitressin, mfg. by Parke, Davis & Co., Detroit, Mich.

The toads are force-fed chopped raw beef liver once a week. During the period of this study (January-June), they appeared to remain healthy and many gained weight on this regime. No seasonal variations in response of the toads to hydration or hormone injection were noted during this period.

The present assay procedure has been used to determine levels of antidiuretic substance in rat plasma under a variety of conditions.[3] Preliminary studies have been conducted on human plasma. Work is in progress on dose-response curves for several other species of North American toads, in the hope of finding another species which may be even more senstivie to vasopressin, or more readily obtainable, or both.

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In the December 1958 issue of Endocrinology four manuscripts listed below should bear date of receipt as follows:

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[3] A full account of this work will be published elsewhere.

CORRECTIONS

In the December 1958 issue of Endocrinology four manuscripts listed below should bear date of receipt as follows:

Bernstein, Donald E.
Autotransplantation of the Adrenal of the Rat to the Portal Circulation: Effect of Administration of Sex Hormones, 765. Received May 6, 1958

Evans, Edward S., Miriam E. Simpson and Herbert M. Evans
The Role of Growth Hormone in Calorigenesis and Thyroid Function, 836. Received June 2, 1958

Yates, Eugene F., Arthur L. Herbst and John Urquhart
Sex Difference in Rate of Ring A Reduction of Δ^4-3-Keto-Steroids *In vitro* by Rat Liver, 887. Received June 12, 1958

Moltke, Erik
Effect of Thyroxine and a Dehalogenase-Inhibitor on the Tensile Strength of Healing Wounds, 931. Received June 6, 1958

THE AMERICAN SOCIETY FOR THE STUDY OF STERILITY

The Fifteenth Annual Meeting of The American Society for The Study of Sterility will be held at The Shelburne Hotel, Atlantic City, N.J., April 3–5, 1959. Persons interested in attending this meeting should apply for advance registration and a complete program to the Secretary, Dr. Herbert H. Thomas, 920 South 19th Street, Birmingham 5, Alabama.

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ELIMINATION OF C^{14}-LABELED THYROXINE IN THE BILE, URINE AND EXPIRED AIR OF RATS IN ALTERED THYROID STATES[1]

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ABSTRACT

Adult male Sprague-Dawley rats were treated with appropriate measures to attain the desired metabolic states. DL-thyroxine-1-C^{14} was administered subcutaneously and the elimination of radioactivity determined in bile, urine, and expired air of hypo-, hyper- and euthyroid animals.

Biliary elimination of C^{14} activity in the control rats amounted to 11.8% of the injected dose within a period of six hours. Desiccated thyroid treated animals showed an increased excretion of radioactivity accompanied with an increased volume of bile produced. The thyroidectomized and thiouracil-treated rats showed no change from normal in the amount of radioactivity eliminated but both groups had a reduction in the volume of bile removed.

Urinary excretion of thyroxine activity during the twelve hour period never exceeded 2% of the injected amount regardless of the group studied. Hyperthyroid animals showed increased urinary excretion of radioactivity with increased volume of urine eliminated. The C^{14} activity in the urine of the thyroidectomized and thiouracil-treated groups was lower than the control animals but only significantly so in the latter case. This reduction in the amount of activity was found to be independent of the urine volume excreted.

The recovery of C^{14} in the expired air was taken to indicate that decarboxylation of the administered thyroxine had occurred. Within twelve hours about 12% of the injected activity appeared as $C^{14}O_2$ in the control group. No alteration in this amount was observed in the hyperthyroid animals. Both hypometabolic groups showed decreased $C^{14}O_2$ production, however, this was only significant in the thiouracil-treated animals.

WITHIN the last two decades I^{131}-labeled thyroxine has been used extensively in studying the degradation and elimination of the thyroxine molecule in both animals and humans. Because the detection of I^{131} activity does not always indicate the presence of the intact thyroxine

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molecule without further chemical analysis, abnormally high levels of excretion for this compound have appeared in the literature.

With the synthesis of C^{14}-carboxyl labeled thyroxine (1), it is now possible to make use of another radioactive element in following the metabolism of the thyroxine molecule; the use of this element furthermore affords an opportunity to study an additional mechanism in the degradation of this compound, namely that of decarboxylation.

The present study was carried out primarily to compare our results with those previously reported using I^{131}-labeled thyroxine, and secondly, to investigate the effect of altered metabolic states on the metabolism of DL-thyroxine-1-C^{14}.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were used throughout the investigation. Animals were divided into four groups comprising a normal, a hyperthyroid and two hypothyroid groups and were treated accordingly to attain the desired metabolic status.

Normal animals were maintained on a Rockland Complete Rat Diet. A hypermetabolic rate was achieved from a diet consisting of 3 gm. desiccated thyroid/Kg. of finely ground stock food. *Ad libitum* feeding on this regime for 3 weeks resulted in the achievement of the desired hypermetabolic level.

For thiouracial feeding, the animals were fed a diet containing the drug concentration of 0.2 gm.%. Animals were maintained on this regime until the metabolic rate decreased about 25%.

Thyroidectomies were performed under ether anesthesia and the animals were maintained on stock diet until ready for use.

A Benedict-Roth closed circuit apparatus was employed for the measurement of oxygen consumption. Basal metabolic rates were obtained by measuring oxygen uptake over three consecutive thirty-minute intervals. Resting metabolic rates of the control group ranged from 90 to 110 cc. O_2/100 gm./hr. An increase or decrease of 25% in oxygen consumption above or below the control levels was used as the criterion for designating animals hyper or hypometabolic.

DL-thyroxine-1-C^{14}, which was used throughout this investigation,[2] had a specific activity of 5.3 μg./mg. All radioactivity measurements were corrected for self-absorption and background and converted into μgm. of thyroxine, by reference to the known specific activity of the administered C^{14} thyroxine. Samples were counted to within a probable error of 3 to 4% in the bile study and to within 6 to 7% in the $C^{14}O_2$ and urine studies. By way of illustration a 200 gram rat received about 840,000 cts./min. and a 0.1 ml. bile sample would have about 1000 cts./min. or 50 times background. All injections were made subcutaneously at a dose level of 780 μg./Kg. body weight. Because of the problem of counting the weak beta radiation from carbon 14, a pharmacological rather than physiological dose was employed in this study. At this dose level certain alterations in the metabolism of the excretory tissues may have occurred if the study had been extended for a longer period of time. However, because this study was limited to 6 hrs. for the collection of bile and 12 hrs. for the urine and $C^{14}O_2$ collections any changes in the metabolism of the excretory tissues was probably not a result of the administered dose of labeled material but by previous treatment.

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[2] The C^{14} for the synthesis of the thyroxine was obtained on allocation from the U.S. Atomic Energy Commission.

Bile samples were obtained by cannulating the common bile duct with polyethylene tubing (inside diameter 0.028 cm.). When a good rate of flow was obtained, the animal was placed in a restraining cage and injected with labeled thyroxine. Aliquots of 0.1 ml. of hourly bile samples were plated in duplicate on copper planchets and diluted with distilled water and 95% alcohol to obtain even distribution of the material and were then allowed to air dry.

For 12-hour urine collections, animals were placed in metabolic cages. The concentration of C^{14} activity in the urine was determined on 0.1 ml. aliquots of samples obtained at 3 hour intervals. The techniques involved in plating, drying, and counting were as described for bile.

Carbon dioxide collections were made using the modified Benedict-Roth closed circuit apparatus (2). The collections were made at 2 to 3 hour intervals through a period of 12 hours. The quantity of radioactivity in each aliquot was then determined using a windowless flow counter.

RESULTS

Table 1 shows the percentage of C^{14}-activity excreted and volume of bile produced. At the end of 6 hours normal animals excreted 11.80% of the injected dose of radioactivity via the biliary route. The hypothyroid groups did not differ significantly from the normal in this respect. Mean excretion values of C^{14}-activity in the thyroidectomized and thiouracil treated animals were 12.04% ($P=0.953$) and 11.90% ($P=0.940$) respectively. A decrease in the volume of bile produced was shown by the thyroidectomized group while no significant alteration in volume was seen in the thiouracil-treated animals.

The amount of radioactivity excreted in the bile of animals treated with desiccated thyroid was significantly elevated ($P=0.001$) above normal, reaching a level of 21.66%, 6 hours after injection. Associated with this increased elimination of radioactivity was a rise in bile production to a level 1½ times greater than normal.

In Table 2 are presented the percentages of injected radioactivity excreted and volume of urine produced for the various groups. In neither group was the level of urine radioactivity above 2% at the end of a 12-hour interval. Normal animals excreted 1.15% of the injected C^{14} activity in 6.22 ml. of urine during the 12-hour collection period. The thiouracil

TABLE 1. CUMULATIVE 6-HOUR BILIARY EXCRETION FOLLOWING *SUBCUTANEOUS INJECTION OF C^{14}-LABELED THYROXINE*

Condition	No. of animals	% activity excreted[a]	P[b]	ml. bile excreted	Mean BMR (cc. O_2/100 gm./hr.)
Normal	7	11.80 ± 3.42	—	4.31	90–110[c]
Desiccated Thyroid-Treated	6	21.66 ± 3.52	0.001	7.10	169.9
Thyroidectomized	6	12.04 ± 9.07	0.953	2.73	57.9
Thiouracil-Treated	9	11.90 ± 2.09	0.945	3.96	68.6

[a] *Mean ± standard deviation.*

[b] *Probability of no difference from normal.*

[c] Range of values for normal animals.

TABLE 2. CUMULATIVE TWELVE-HOUR URINARY EXCRETION FOLLOWING SUBCUTANEOUS INJECTION OF C^{14}-LABELED THYROXINE

Condition	No. of animals	% activity excreted[a]	P[b]	ml. urine excreted	Mean BMR (cc. O_2/100 gm./hr.)
Normal	10	1.15 ± 0.25	—	6.22	90–110[c]
Desiccated Thyroid-Treated	8	1.89 ± 0.40	0.001	11.95	154.8
Thyroidectomized	6	1.07 ± 0.57	0.688	6.4	68.1
Thiouracil-Treated	9	0.79 ± 0.31	0.008	6.02	64.4

[a] Mean ± standard deviation.
[b] Probability of no difference from normal.
[c] Range of values for normal animals.

treated group showed a significant decrease in the amount of radioactivity eliminated ($P = 0.008$) while the thyroidectomized group did not ($P = 0.688$.) The quantity of radioactivity appearing in the urine of hyperthyroid animals was significantly increased to 1.89% ($P = 0.001$). The volume of urine produced by the hypermetabolic group was almost double (11.9 ml.) that of the normal (6.2 ml.) while neither of the hypothyroid groups showed a noticeable change in urine volume when compared with the control group.

In Table 3 are presented the mean cumulative percentages of $C^{14}O_2$ in the expired air by the various groups. This table shows that both hypometabolic groups excreted less $C^{14}O_2$ in 12 hours than the euthyroid animals. Thyroidectomized and thiouracil treated animals show a mean excretion of 4.04 and 2.67%, respectively, with a significant decrease ($P = 0.006$) present in the latter group only. During the 12 hour collection period hypermetabolic animals exhibited no alteration ($P = 0.992$) in eliminating $C^{14}O_2$ when compared with the euthyroid group.

DISCUSSION

The fact that the liver plays an important role in the metabolism of thyroxine is born out by the predominance of radioactivity appearing in the bile. The mean excretion of 11.80% of the C^{14}-activity in the bile of normal animals agrees closely with the results reported earlier when using I^{131} labeled L-thyroxine (3) and C^{14}-labeled DL-thyroxine (2). Other in-

TABLE 3. CUMULATIVE TWELVE-HOUR $C^{14}O_2$ EXCRETION FOLLOWING SUBCUTANEOUS INJECTION OF C^{14}-LABELED THYROXINE

Condition	No. of animals	% activity excreted[a]	P[b]	Mean BMR (cc. O_2/100 gm./hr.)
Normal	6	6.18 ± 2.39	—	90–110[c]
Desiccated Thyroid-Treated	8	6.20 ± 2.47	0.992	186.6
Thyroidectomized	5	4.04 ± 0.78	0.288	71.8
Thiouracil-Treated	7	2.67 ± 1.26	0.006	68.6

[a] Mean ± standard deviation.
[b] Probability of no difference from normal.
[c] Range of values for normal animals.

vestigators (4) gave a comparable value of about 13% excretion via this route in 6 hours using tracer amounts of L-thyroxine I^{131}. It was reported (5), that much of the I^{131} which appeared in the bile was in the form of a glucuronide of thyroxine.

The marked increase in the biliary radioactivity of hypermetabolic animals may be due to increased liver metabolism. A previous report(3) indicated that there was no significant increase in I^{131} activity in the bile of hyperthyroid animals having been injected with L-thyroxine. One possible explanation for this discrepancy may be due to the difference in turnover rates of the L and D isomers of thyroxine. It has been reported (6) that after a physiological or pharmacological dose of L-thyroxine the resulting iodinemia is reduced to 1 to 2% of the initial level within 2 hours. Also the disappearance from the blood of both of the thyroxine isomers have been reported (7) to be composed of five exponential decay rates which can be reduced to four by drainage of the bile to the outside or removal of the thyroid gland. The first three rates are the same for both isomers, however, the half life of the final rate for the L-isomer is between 2 to 3 times that for the D form indicating a more rapid turnover of the D isomer. Other investigations have shown that the metabolism of a thyroidectomized rat can be maintained at its normal level by the administration of 12 μg. DL-thyroxine per Kg. of body weight/day (8), while only 6 μg. of L-thyroxine is required per Kg. per day (9).

Although there is a diminution of the bile production by hypothyroid animals, this appears to be only a characteristic of the hypometabolic state and is not reflected in the amount of radioactivity excreted.

Apparently thyroidectomized and thiouracil-treated animals suffer no loss in the ability of the liver to metabolize exogenous thyroxine at this dose level although bile production is reduced. It has been observed (10) that hyperthyroid animals produce approximately twice the volume of bile per day when compared with hypothyroid animals. Such a relationship was also found in the present study.

The urinary pathway appears to play a minor role as a route of excretion for the C^{14}-labeled thyroxine molecule per se. Although the percentage of radioactivity excreted in the urine was low in all groups, there was a statistically significant increase above the normal level of 1.15% found in the hyperthyroid animals after 12 hours. C^{14}-activity in the urine of both thyroidectomized and thiouracil-treated animals was lower than the control group but only significantly so in the latter.

Earlier reports (6, 11) indicated that in a period of 24 hours normal rats excreted between 11.0% and 18.2% of the injected I^{131}-labeled thyroxine in the urine. Analysis of the urine samples showed the majority of the I^{131} to be in the form of inorganic iodide. Intact, radioactive thyroxine usually appeared in amounts of less than 5% of the total I^{131} excreted in the urine.

The apparent discrepancy that exists with regard to the quantity of radioactivity appearing in the urine following administration of C^{14}-labeled and I^{131}-labeled thyroxine is probably the result of large quantities of I^{131} being excreted as inorganic iodide resulting from metabolized I^{131}-labeled thyroxine and not representative of the intact thyroxine molecule.

Although the differences in the amount of radioactivity recovered in the urine of the hyperthyroid and thiouracil treated animals were small when compared to the control group, the values obtained were highly significant ($P = 0.001$ and $P = 0.008$, respectively). The observed differences are likely due to changes in metabolism, which allows increasing or decreasing amounts of available metabolites to be excreted. Thyroidectomy appears to have no effect on the excretion of thyroxine or its metabolites in the urine. Urine production itself does not seem to affect the amount of radioactivity that will be excreted by this route.

The recovery of C^{14} in the expired air indicates that catabolism of the thyroxine molecule has taken place in the body, and that this pathway may be an important route for the elimination of thyroxine metabolites.

Both hypometabolic groups show a decrease in elimination of $C^{14}O_2$ as shown in Table 3. There is, however, a significant alteration only among the thiouracil-treated animals. It would seem safe to conclude that the rate of decarboxylation of the thyroxine molecule is decreased with lowering of the metabolic state of the animal below a critical level of about 70cc. O_2/100gm./hr.

The hypermetabolic animals and the control group eliminated nearly identical amounts of $C^{14}O_2$ after 12 hours. A much higher rate of $C^{14}O_2$ excretion was previously reported in normal animals (2). After a similar time interval they found 10% of the injected C^{14} activity to be eliminated by this route as compared to 6.2% in this report.

The fact that hyperthyroid animals should show no increase in $C^{14}O_2$ may be due to one of two factors. The high biliary elimination of C^{14} activity may have lowered the amount of available thyroxine or its metabolites to be eliminated by other routes or with an increased body metabolism there may have been little or no increase in the amount of the decarboxylating enzymes.

In summarizing the differences found in various metabolic states the hyperthyroid animals showed an increased biliary excretion, of what may be conjugated thyroxine with an increased volume of bile produced. There was a small but significant increase in urinary excretion of thyroxine, but no significant alteration in the rate of decarboxylation of thyroxine as measured by the appearance of $C^{14}O_2$ in the expired air.

Neither of the hypometabolic groups showed an alteration in the amount of thyroxine eliminated in the bile although both groups had a reduction in the volume of bile produced. In both groups the urinary excretion was reduced, but only significantly in the thiouracil-treated animals, with no significant change in the volume of urine eliminated. Reduced decarboxylation of thyroxine was found in both hypometabolic groups although this was only highly significant in those animals receiving thiouracil.

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EFFECT OF THYROID IMBALANCE ON THE DETOXICATION OF BENZOIC ACID IN THE RAT

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ABSTRACT

Thyroid hormone imbalance has been shown to profoundly influence the detoxication of benzoic acid in the rat. The excretion of hippuric acid and benzoylglucuronide following the administration of sodium benzoate is low in hyperthyroid rats when compared with normal. *In vitro* studies with tissue slices have confirmed the results obtained with intact rats. The thyroid status of the animal has also been found to influence the utilization of administered glucuronolactone. The β-glucuronidase activity of hypothyroid rat livers is depressed below normal and the extent of glucosiduronic acid synthesis in these livers is much higher than normal. The various implications of these results are discussed.

THE study of the detoxication as well as the metabolism of foreign organic substances in the animal body has, in recent years, developed into an elegant and recognised method of investigating the mechanism of several synthetic processes in the body. Of the diverse metabolic reactions that are altered under thyroid stress, the influence of thyroid hormone on oxidative phosphorylation has received critical attention in recent years. Though Tapley, Cooper and Lehninger (1) have recently observed that *in vitro*, thyroxine has no direct influence on oxidative phosphorylation processes, the same authors and several others (2, 3, 4) have shown that thyroid hormone *in vivo* depresses oxidative phosphorylation. Ball and Cooper (5) have recently outlined the probable mechanism whereby, in hyperthyroidism, phosphorylation would be depressed and oxygen consumption enhanced.

Benzoic acid is normally metabolized in the body to form hippuric acid, a glycine conjugate, and also as benzoylgucuronide. Recent investigations have shown that hippuric acid synthesis (6) and glucuronide conjugation (7) are both dependent on high energy phosphates. Burlington (8) studied the influence of endocrines on p-amino hippuric acid synthesis by rat liver homogenates and showed that the ability of the homogenates to synthesize p-amino hippuric acid is altered by adrenalectomy, a condition of alloxan diabetes (representing the influence of insulin lack) and

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ovariectomy. Fatterpaker, Marfatia and Sreenivasan (9) have observed that feeding iodinated casein to rats reduces the hippuric acid synthesis by liver homogenates.

A detailed investigation into the influence of thyroid stress on benzoic acid detoxication has revealed the two endergonic processes—hippuric acid and benzoylglucuronide synthesis—to be profoundly influenced by the thyroid status of the animal. The present study also provides indirect evidence for the participation of high energy phosphate (uridine diphosphate glucuronic acid) in the synthesis of glucosiduronic acids.

EXPERIMENTAL

Animals:

Albino rats weighing between 150 and 160 gm. were used in the present series of experiments. Each series consisted of rats divided into 3 groups: the control group fed the laboratory stock diet, the hypothyroidal group fed the stock diet plus a supplement of 50 mg. of methylthiouracil per rat per day, and the hyperthyroid group fed the stock diet plus 16 mg. of desiccated thyroid power (B.D.H.) per rat per day. In each set of experiments, a total of not less than fifteen rats was used. The diets were fed *ad libitum* and sufficient distilled water was given. Vitamin A and D were given as a commercial preparation. Each rat received per week 7200 I.U. of vitamin A, 1200 I.U. of vitamin D, 2 mg. of α-tocopherol and 0.2 mg. of vitamin K. From preliminary experiments (10) it was observed that for hypo- and hyperthyroidal conditions to set in completely, a six-week period of feeding on the above regimen was necessary.

Benzoic acid detoxication:

In preliminary experiments reported earlier (10), benzoic acid was fed orally to rats. In the present study, however, it was found convenient to administer benzoic acid by intraperitoneal injection as sodium benzoate. Each rat received a single injection of 50 mg. of benzoic acid. The rats were transferred to metabolism cages and the urine of the subsequent 48 hours was collected in 1 ml. of 1N H_2SO_4 to which a few drops of toluene were added as a preservative. The urine sample in each case was made to 100 ml., filtered and preserved under toluene in an ice-chest.

Hippuric acid in the samples was estimated by the paper chromatographic procedure of Gaffney, Schreier, Di Ferrante and Altman (11). 100 μl. of the urine samples and known amounts of pure hippuric acid were chromatographed on Whatman No. 1 paper in the solvent system, n-butanol:glacial acetic acid:water (4:1:1 by volume). After drying, the chromatogram was sprayed with a 4% solution of p-dimethylaminobenzaldehyde in acetic anhydride containing a few crystals of sodium acetate, and again dried at 130° C. The orange-colored hippuric acid-azlactone spots (R_f 0.80) were then cut out and eluted thrice with 2 ml. lots of methanol. The intensity of the color was measured at 460 mμ in a Beckman model DU spectrophotometer.

Benzoylglucuronide in the urine samples was estimated by the method of Bray, Humphris, Thorpe, White and Wood (12). Suitable aliquots were transferred to boiling tubes, the volume in each tube was made to 2 ml. and to each tube were added 4 ml. of conc.HCl and 2 ml. of 0.375% naphthoresorcinol solution. The tubes were heated in a boiling water bath for 2 hours. After cooling, 5 ml. of n-butanol was added to each tube and the contents mixed well. After about 2–3 minutes the color was read in a Lumetron Photoelectric Colorimeter (Photovolt Corporation) employing yellow-green (530 mμ) filter.

Metabolism of glucuronolactone:

This experiment was conducted with a fresh set of rats rendered hypo- and hyperthyroidal as above. Each rat after an intraperitoneal injection of 100 mg. of glucuronolactone was transferred to a metabolism cage and the urine collected during the next 24 hours in 2 ml. of 1N H_2SO_4 to which a few drops of toluene were added. The total glucuronic acid was estimated according to the method of Bray and coworkers (vide supra).

In vitro synthesis of ortho-aminophenol glucuronide (OAP-glucuronide) by liver slices:

The same set of rats used for the above experiment was used in the present investigation. After the injection of glucuronolactone, a lapse of 10–15 days was allowed for the rats to reach their respective normal status, after which they were killed. The method followed for glucuronide synthesis was essentially that of Levvy and Storey (13). The reaction was carried out in 25 ml. conical flasks in a Dubnoff metabolic shaking incubator for a period of 90 minutes at a bath temperature of 37° C. The composition of the reaction medium is given in detail in Table 4. After removing the slices for dry weight and nitrogen determination, 2 ml. of a fresh mixture of equal volumes molar trichloroacetic acid and molar phosphate was added to the flasks. After centrifuging, suitable aliquots of the supernatant were removed for color development by diazotisation and coupling with naphthethylene diamine dihydrochloride. The color complex was measured in a Beckman spectrophotometer (model DU) at 550 mμ. Each liver sample was tested in triplicate to determine its ability to synthesize OAP-glucuronide.

Liver β-glucuronidase activity:

The enzyme extract from the individual liver samples was prepared according to the method of Kerr, Graham and Levvy (14). To a cell-free liver homogenate (1%) was added 0.3 *M* citrate buffer pH 5.2 to remove the inactive proteins and the supernatant after centrifugation was treated with an equal volume of saturated ammonium sulfate solution, centrifuged again and the residue dissolved in water. This solution was treated as the enzyme source.

The incubation mixture for β-glucuronidase assay contained 4 ml. of 0.1 *M* acetate buffer pH 4.5, 0.5 ml. of 0.01 *M* sodium phenolphthalein glucuronide (in the control the substrate was added at the termination of the incubation period) and 0.5 ml. of the appropriate enzyme solution. The incubation was carried out at 37° C for a period of 60 minutes. The reaction was stopped by the addition of 5 ml. of 0.4 *M* glycine buffer pH 10.45. The intensity of the color formed due to the liberation of free phenolphthalein by the action of the enzyme was measured in a Lumetron colorimeter using filter 530 mμ. The substrate consisting of 0.01 *M* sodium phenolphthalein glucuronide was prepared from the pure cinchonidine salt of phenolphthalein monoglucuronide according to the method of Talalay *et al.* (15).

RESULTS

In Table 1 are presented data regarding the excretion of hippuric acid and benzoyl glucuronide, by rats under thyroid stress, following an intraperitoneal injection of 50 mg. of benzoic acid. It can be seen that the excretion of both hippuric acid and benzoyl glucuronide is depressed to a marked extent in hyperthyroidal rats, whereas it is enhanced in hypothyroidal animals. The resuls presented in Table 2 give a comprehensive picture of the extent of detoxication of benzoic acid in various states of

TABLE 1. INFLUENCE OF THYROID HORMONE ON HIPPURIC ACID AND BENZOYLGLUCURONIDE EXCRETION IN THE RAT

Condition	No. of rats	Hippuric acid excretion* (mg.)	Percent deviation from normal	Benzoyl glucuronide excretion* (mg.)	Percent deviation from normal
Normal	5	27.7 ± 1.4† (26.6 – 30.6)	—	16.6 ± 1.9† (13.5 – 20.0)	—
Hypothyroid	5	40.8 ± 8.0 (28.0 – 63.9)	+47	22.4 ± 4.5 (18.0 – 36.5)	+35
Hyperthyroid	5	13.1 ± 1.8 (11.0 – 16.7)	–53	10.2 ± 2.0 (7.3 – 12.3)	–39

* The values in parenthesis indicate range.
† Standard deviation.

TABLE 2. INFLUENCE OF THYROID HORMONE ON DETOXICATION OF BENZOIC ACID

Condition	Hippuric acid expressed as benzoic acid (mg.)	Benzoyl glucuronide expressed as benzoic acid (mg.)	Total amount of benzoic acid derivatives excreted (mg.)	Percent of injected benzoic acid excreted as conjugates
Normal	18.86	6.80	25.66	51
Hypothyroid	27.90	9.17	37.07	74
Hyperthyroid	8.93	4.17	13.10	26

TABLE 3. METABOLISM OF GLUCURONOLACTONE IN NORMAL, HYPO- AND HYPERTHYROIDAL RATS
(100 mg. of glucuronolactone injected intraperitoneally)

Condition	Number of rats	Glucuronic acid excreted in 24 hours (mg.)		Per cent deviation from normal
		Range	Mean	
Normal	5	28–56	35 ± 7.2*	—
Hypothyroid	5	18–38	26 ± 7.4	–26
Hyperthyroid	5	45–64	53 ± 5.7	+51

* Standard deviation.

thyroid stress, the amounts of benzoic acid conjugates excreted being expressed in terms of benzoic acid.

The influence of thyroid hormone on glucuronide conjugation was considered worth studying in detail, since it is known that both thyroxine and triiodothyronine are excreted from the liver as glucuronide conjugates via the bile into the intestinal tract (16). In Table 3 is shown the excretory pattern of total glucuronic acid following an intraperitoneal injection of 100 mg. of glucuronolactone to the three groups of rats. The results show that the excretion is depressed considerably in hypothyroidism, whereas, it is enhanced by an appreciable extent in hyperthyroidism.

TABLE 4. EFFECT OF THYROID HORMONE IMBALANCE ON 'IN VITRO' SYNTHESIS OF O-AMINOPHENOL GLUCURONIDE IN RAT LIVER SLICES

The reaction medium was of the following composition: 2 ml of bicarbonate-Ringer medium containing 0.02 *M* lactate, 0.001 *M* ascorbic acid and 0.0025% o-aminophenol. The bicarbonate-Ringer medium did not contain any sulphate as suggested by Levvy and Storey (14). Liver slices of 20 mg. wet weight (approximately) per flask. Incubation for a period of 90 minutes at 37° C. Gas phase 95% oxygen and 5% carbon dioxide.

Condition	Number of rats	O-aminophenol glucuronide synthesized per mg. nitrogen of liver tissue in 90 minutes (μg.) Range	Mean	Per cent deviation from normal
Normal	5	20.87–29.66	24.64 ± 3.0*	—
Hypothyroid	5	30.50–37.20	33.48 ± 2.4	+36
Hyperthyroid	5	18.90–20.30	19.80 ± 0.5	−20

* Standard deviation.

The results presented in Table 4 show that the *in vitro* synthesis of OAP-glucuronide by liver slices of rats under thyroid stress, follows the same pattern as the *in vivo* synthesis of benzoyl glucuronide. In Table 5 are summarized the results of assay for β-glucuronidase activity in liver samples of eu-, hypo- and hyperthyroid rats. The enzyme activity is considerably enhanced in hyperthyroidism, whereas, it is decreased in hypothyroidism.

DISCUSSION

The work of Chantrenne (17) and more recently that of Schachter and Taggart (18) have demonstrated the essential nature of adenosinetriphosphate (ATP) in the formation of benzoyl coenzyme A, the active coupling agent in the hippuric acid conjugating system. Dutton and Storey (19) and Dutton (20) have similarly shown uridinediphosphate glucuronic acid (UDPGA), a high energy phosphate compound to be an active participant in the synthesis of ether- and ester-linked glucuronides. Thus, a decreased synthesis of benzoylglucuronide and benzoylglycine in hyperthyroidism is to be expected, if, as suggested by recent workers (2–5), the hyperthyroidal state is associated with decreased phosphorylation and a consequent poor availability of high energy phosphate compounds.

TABLE 5. β-GLUCURONIDASE ACTIVITY OF LIVERS OF NORMAL, HYPO- AND HYPERTHYROIDAL RATS

Condition	Number of rats	β-glucuronidase activity per gm. of liver* Range	Mean	Per cent deviation from normal
Normal	4	3.94–5.40	4.71 ± 0.47†	—
Hypothyroid	5	2.51–3.34	3.07 ± 0.21	−35
Hyperthyroid	5	6.31–8.57	7.65 ± 0.59	+62

* Arbitrary unit of β-glucuronidase is defined as mg. of phenolphthalein liberated from phenolphthalein glucuronide in an hour at 37° C by 1 gm. of fresh liver tissue.

† Standard deviation.

Hyperthyroidism is associated with an increased catabolism of carbohydrate and in this condition tissue vitamin C is decreased (21, 22) and the requirement for vitamin C is greater (23). The opposite has been shown to hold for hypothyroidism (21). Glucuronic acid is known to be a common precursor for both ascorbic acid and glucosiduronic acid. Since carbohydrate metabolism proceeds at different rates in the various states of thyroid stress, the excretory pattern of glucuronolactone indicated in Table 3 may well reflect the extent of utilization of the same for various metabolic needs of the body. The results presented in Table 3 are in keeping with the enhanced synthesis of benzoylglucuronide observed in hypothyroidal rats in a separate experiment (Table 1) and would seem to suggest that in hypothyroidal condition, the utilization of glucuronolactone is better than in normal, the retained amount being utilized at least in part for the formation of increased amounts of tissue vitamin C and the glucuronide conjugates. On the contrary, in hyperthyroidism the utilization seems to be poor, even the glucuronic acid retained in the system probably being mainly catabolized. Thus, the present results suggest that the lowered excretion of glucuronide observed in hyperthyroidism may be the result of *the combined effect of a reduced availability and lowered mobilization of glucuronic acid for conjugation and a decreased rate of conjugation due to the lack of high energy phosphate compounds*. The pattern of results obtained in the course of the study of the *in vitro* synthesis of OAP-glucuronide by liver slices of rats under thyroid stress (Table 4) confirms the *in vivo* observation.

Dutton and Storey (19) and Isselbacher and Axelrod (24) hold that glucuronic acid in the form of UDPGA conjugates with an acceptor molecule in the presence of an enzyme (which does not resemble β-glucuronidase in its properties) from cell-free extracts of rat liver. On the other hand, Fishman and Green (25) contend that β-glucuronidase catalyses efficiently the transfer of glucuronic acid from a conjugate to a suitable acceptor molecule. Since sufficient experimental evidence is available to support both the postulates, it was considered of interest to assay the liver samples of the three groups of rats for β-glucuronidase activity and the results are summarized in Table 5. The increase in the β-glucuronidase activity in the livers of hyperthyroid rats when considered with the observed decrease in the glucosiduronic acid synthesis by liver slices of hyperthyroid rats, clearly shows that *in this tissue at least*, β-glucuronidase may not be associated with glucosiduronic acid synthesis. The opposite holds true for hypothyroidal rats. Since the physiological function of β-glucuronidase apart from its being associated with the hydrolysis of glucuronides, is not well understood, it is not possible at this stage to offer any satisfactory explanation for the increase or decrease in β-glucuronidase activity observed in positive and negative thyroid stress respectively.

It is clear from the results presented in Table 2 that the overall detoxication of benzoic acid is much more efficient in hypothyroidal condition than in normal, whereas it is poor in hyperthyroidal condition. In the light of the results of the present investigation, it would be of interest to study the influence of thyroid stress on the detoxication of organic compounds like phenols, naphthalenes etc.

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THYROID FUNCTION IN EXPERIMENTAL NEPHROTIC SYNDROME[1]

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ABSTRACT

Thyroid function and thyroid hormone metabolism have been investigated in the experimental nephrotic syndrome produced by anti-kidney antibodies. Nephrotic animals differed from normal in three ways: (1) thyroxine was lost in the urine in conjunction with protein, to the extent of 11 ± 7% of a tracer dose; (2) the response to pharmacological doses of thyroxine, as determined by increase in oxygen consumption rate, was decreased by about 30%; (3) the rates of iodine uptake and of thyroxine secretion by the thyroid were increased, the latter by at least 40%. Since the response of the oxygen consumption rate to triiodothyronine was also decreased by about 30% even though this substance was not excreted to a significant extent in the urine, it was concluded that the first two changes represented primary abnormalities in iodine metabolism resulting from the nephrotic state and that the last was a compensatory change.

THE occurrence of lowered basal metabolic rate and elevated serum cholesterol in patients with the nephrotic syndrome led to the suggestion that such patients have a metabolic disturbance allied to hypothyroidism (1). This view received support from the later observation of diminished serum protein-bound iodine concentration in nephrotic patients (2). Recent studies have indicated that thyroidal uptake of iodine is in fact normal or increased in this condition, but there have been conflicting reports about the state of thyroxine metabolism (3, 4). Since some of the latter discrepancies may have been due to variability among the subjects with regard to degree of proteinuria, edema and iodine intake, the problem of thyroid-nephrosis interrelationships has been studied in the rat made nephrotic with anti-kidney serum. Several weeks after injection of serum, such animals have proteinuria, hypoalbuminemia and hyperlipemia, but no edema.

METHODS

Adult male rats maintained under constant environmental conditions (77–79° F and 40–42% relative humidity with constant lighting from 8 A.M. to 8 P.M.) were employed for all studies. Three types of diet were used: (a) Purina fox chow, (b) synthetic stock diet, high in iodine, (c) synthetic low-iodine diet of the same composition as (b) but

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having an iodine content of approximately 140 μg./kg. The synthetic diets were given by gavage or by *ad libitum* feeding; the former method provided a daily iodine intake of about 3 μg. in the case of the low-iodine diet.

Thyroidal radioactivity was calculated from the epithyroid count after correction for extrathyroidal neck activity (5). Urine was assayed in a scintillation well counter for total and protein-bound (insoluble in 10% trichloroacetic acid) radioactivity. Oxygen consumption was measured over fifteen-minute periods in an apparatus as described by Holtkamp *et al.* (6). Paper strip electrophoresis was carried out in veronal buffer at pH 8.6, ionic strength 0.075, at 120 volts for 15 hours. For localization of radioactivity, the paper strips were stained with bromphenol blue, scanned and then cut into segments 3 mm. wide. Each segment was counted in a scintillation well counter, and from the results a curve drawn of the distribution of radioactivity.

Iodine determinations were performed on thyroid glands and serum by the method of Zak *et al.* (7).

Nephrosis was induced by the method of Heymann and Lund (8) 3–4 weeks before the experimental period was started.

RESULTS

Studies of Thyroid Accumulation and Release of I^{131}

Accumulation gradient and thyroidal uptake. Five nephrotic and six control animals previously maintained on a low-iodine diet fed by gavage for 10 days were given 10 μc. of carrier-free NaI^{131} intraperitoneally; epithyroid counting was then performed at hourly intervals for 4 hours, and the accumulation gradients calculated (9). A further count was taken 18 hours after administration of the radioiodine. The results (Table 1) indicate a more rapid accumulation in the nephrotic rats, and a greater uptake at 18 hours.

Thyroidal release and urinary excretion of I^{131}. As a continuation of the above experiment, epithyroid counting was performed at 24-hour intervals, beginning 48 hours after injection of radioiodine. The negative of the

TABLE 1. THYROIDAL ACCUMULATION AND RELEASE OF RADIOIODINE IN NEPHROSIS

	Diet	Control	Nephrotic	P
Accumulation Gradient	Gavage	1.5 ± 0.1 (6)	3.3 ± 0.6 (5)	< .05
Uptake (%)	Gavage	15 ± 2 (6)	32 ± 3 (5)	< .01
	Ad lib.	37 ± 3 (6)	43 ± 2 (6)	< .05
Iodine Release Rate constant	Gavage	0.0338 ± 0.007 (6)	0.0579 ± 0.002 (5)	< .05
	Ad lib.	0.0659 ± 0.016 (6)	0.0863 ± 0.005 (7)	< .05
Per cent of dose 48–144 hours	Gavage		9.9 ± 1.3 (5)	
48–120 hours	Ad lib.		8.6 ± 0.7 (7)	
Thyroidal I^{127} (μg.) at midpoint of experimental period	Gavage	9.0 ± 0.8 (6)	7.5 ± 0.6 (5)	< .01
	Ad lib.	9.5 ± 0.4 (5)	10.0 ± 1.4 (5)	> .05

In this and all subsequent tables, the values shown are Mean ± Standard Error, while the number in parentheses indicates the number of animals used.

regression coefficient of logarithm of thyroidal radioactivity on time was taken as the rate constant of release of hormonal radioiodine. Radioactivity in urine was measured daily and fecal radioactivity was measured on pooled feces for the whole experimental period, for each animal. Immediately after the last neck count, serum concentration of I^{131} and I^{127} and thyroidal I^{131} were measured. Separate groups of normal and nephrotic animals treated in an identical manner were sacrificed at the midpoint of the experimental period and the thyroids analyzed for I^{127}; these values were taken as approximations of the mean thyroidal I^{127} for the experimental period in the main groups. A similar experiment was performed on six normal and seven nephrotic rats, maintained on a low iodine diet fed *ad libitum* for 5 days prior to injection of radioiodine; serum PBI^{131} measurements, however, were not made.

TABLE 2. SERUM PBI AND PBI^{131} IN NEPHROSIS

Experiment		Control	Nephrotic	P
1	PBI^{127} μg./100 ml.	3.0 ± 0.5 (6)	2.3 ± 0.3 (5)	$>.05$
	PBI^{131} % of dose/100 ml.*	3.6 ± 0.9	5.4 ± 0.8	$<.05$
2	PBI^{127} μg./100 ml.	4.9 ± 0.3 (6)	4.5 ± 0.4 (7)	$>.05$

* 144 hours after administration of I^{131}.

In both experiments the release rate constant was higher in the nephrotics (Table 1). The value of (rate constant × thyroidal iodine content) may be taken as an index of the rate of hormone secretion (as expressed for example in micrograms/day). The mean indices so calculated are .304 for control animals and .434 for nephrotics, indicating a mean increase in hormonal secretion in the latter group of about 41%. In the second experiment the mean increase was of the order of 37%.

By comparison with a standard, thyroidal radioactivity measured *in vitro* was calculated as a percentage of the administered dose. From the relationship between the *in vitro* and final *in vivo* counts, release of I^{131} as a percentage of the dose was calculated for the period beginning 48 hours after radioiodine administration (Table 1).

Serum PBI^{127} values were not significantly different in the two groups, but PBI^{131} concentrations were considerably higher in the nephrotics (Table 2). The mean relative specific activities of serum PBI, calculated from these measurements, were 1.23 ± .18% of dose per microgram for control animals, and 2.82 ± .53% per microgram for nephrotics.

The urinary radioactivity is shown (Table 3) for the period beginning 48 hours after administration of I^{131}; it may reasonably be assumed that all inorganic I^{131} excreted after this time was derived from degradation of

TABLE 3. URINARY AND FECAL EXCRETION OF I^{131} AFTER ADMINISTRATION OF RADIOIODINE (% of Dose)

Experiment			Total	Organic	Inorganic*
1 (48–144 hours)	Control (6)	Urine	5.8 ± 0.5	0.06 ± 0.01	5.7
		Feces		5.3 ± 1.3	
	Nephrotic (5)	Urine	11.3 ± 1.4	2.1 ± 0.8	9.2
		Feces		6.1 ± 1.4	
2 (48–120 hours)	Control (6)	Urine	7.6 ± 0.6	0.045 ± 0.01	7.6
		Feces		7.9 ± 2.2	
	Nephrotic (7)	Urine	9.1 ± 0.4	2.1 ± 0.2	7.0
		Feces		9.0 ± 1.1	

* By difference.

labelled hormone. Urinary excretion of organic I^{131} by nephrotic animals was considerably greater than normal. In the two experiments it amounted on the average to 19 ± 6 and 25 ± 4% of the radioiodine released from the thyroid gland, and to 18% and 23% of the total urinary I^{131} (calculated from data in Table 3). There was little PBI^{131} excretion until the proteinuria reached a level of about 300 mg. per day (Fig. 1); as protein excretion increased beyond this level, there was a progressive increase in the excretion of protein-bound I^{131}. The radioiodine content of feces pooled for the entire experimental period for each animal was not significantly different in the two groups.

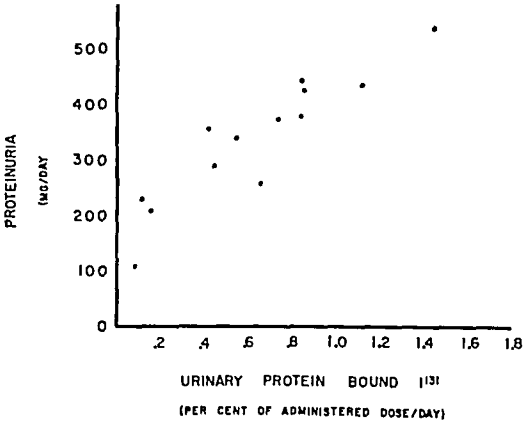


FIG. 1. Relationship between urinary protein and urinary PBI^{131}. Values are the means for the interval 48–120 hours after administration of inorganic I^{131}.

Metabolism of Thyroxine and Triiodothyronine

Control and nephrotic rats were given 0.5 μg. of *l*-thyroxine-I^{131} intravenously and the daily urinary excretion of I^{131} was measured for the next 3 days. Before and during this experiment the animals received a high iodine diet on an *ad libitum* basis to reduce thyroid uptake of I^{131} released by degradation of thyroxine. It may be assumed that by the end of the experimental period virtually all I^{131} had been excreted in the urine or feces (10). The total urinary radioactivity of the two groups did not differ significantly, but the nephrotics excreted much more in a protein-bound form. Twenty-two per cent of the total urinary I^{131} in this group was protein-bound, a figure in good agreement with that of the preceding experiment in which the thyroxine had been endogenously labelled. The total PBI^{131} excreted in 3 days was 11 ±7%. The rate of thyroxine-I^{131} excretion was determined for each animal by extrapolating to zero time a semilogarithmic plot of urinary PBI^{131} (as percentage of dose) on time (days after thyroxine administration). The mean value obtained was 19 ±3% of the dose per day. The relationship between protein and thyroxine excretion in the urine was similar to that previously observed after giving NaI^{131} (Fig. 2).

After administration of 0.07 μg. of *l*-triiodothyronine-I^{131} to four nephrotic rats, and measurement of urinary radioactivity for the next 2 days, it was found that 44.9±2.1% of the administered radioactivity appeared in the urine but only 1.0 ±0.2% of the dose was in protein-bound form. These values are comparable to those obtained in normal rats (20).

When blood and urine were examined electrophoretically after intravenous injection of 0.5 μg. of *l*-thyroxine-I^{131}, the radioactivity in serum was

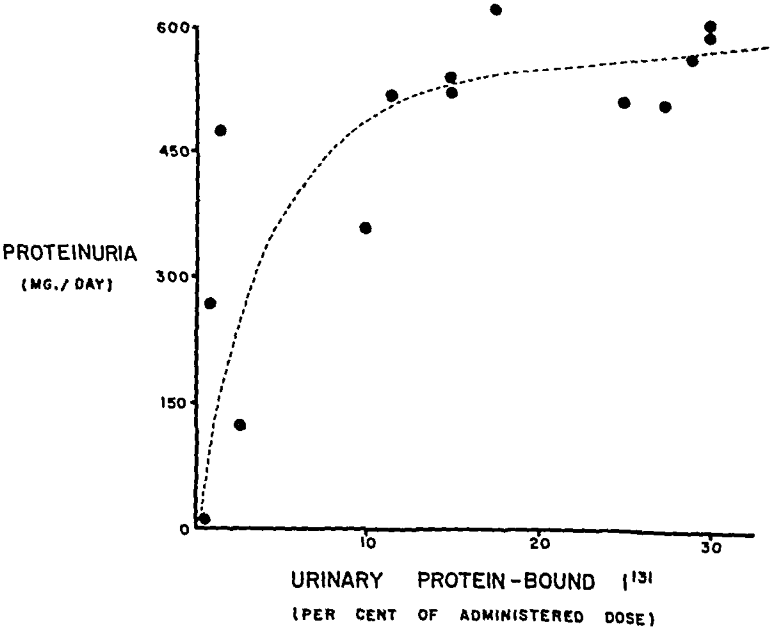


FIG. 2. Relationship between urinary protein and urinary PBI^{131} after administration of *l*-thyroxine-I^{131}. Values for protein are means for the first three days after injection of thyroxine. The values for PBI^{131} represent the rates of excretions in % of dose/day, calculated as described in the text.

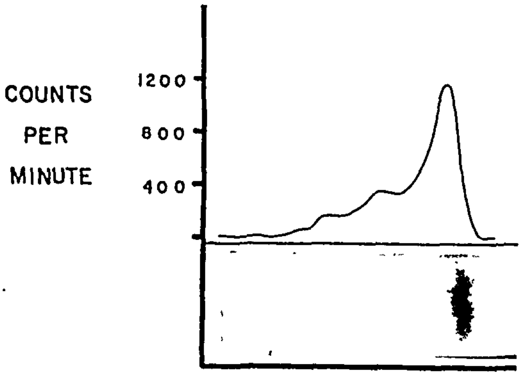


FIG. 3. Distribution of radioactivity of *l*-thyroxine-I^{131} in normal rat serum after electrophoresis.

distributed in two main peaks; one corresponding in mobility to the inter-alpha area, and the other to α_1 globulin or the area between α_1 and albumin. There was a small peak in the β globulin area as well. This pattern was qualitatively similar to that obtained in normal animals. The pattern in the urine differed only in that the front-running peak of radioactivity clearly coincided with the albumin peak (Figs. 3 and 4). Addition of thyroxine-I^{131} to nephrotic urine *in vitro* gave patterns identical to those obtained *in vivo.*

On electrophoretic examination of the serum and urine obtained 30 minutes after injection of 0.07 μg. *l*-triiodothyronine-I^{131} the radioactivity had the expected distribution in serum (11), showing association with the α globulins and extensive trailing in the regions of β and γ globulins; in the urine, however, it was associated largely with albumin.

Effect of Thyroid Hormones on Oxygen Consumption Rate

Oxygen consumption rates were determined before and during the administration of increasing doses of *l*-thyroxine and *l*-triiodothyronine,

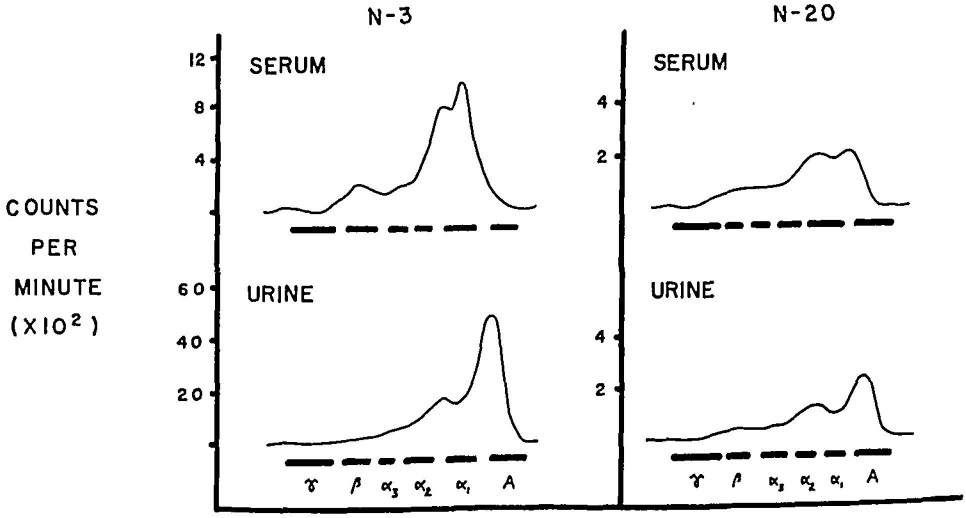


FIG. 4. Distribution of radioactivity in serum and urine of nephrotic rats after injection of *l*-thyroxine-I^{131}.

TABLE 4. URINARY EXCRETION OF I^{131} IN 3 DAYS AFTER ADMINISTRATION OF THYROXINE-I^{131}

	Per cent of Administered Dose	
	Total	Organic
Control (6)	56 ± 5	—
Nephrotic (13)	49 ± 21	11 ± 7

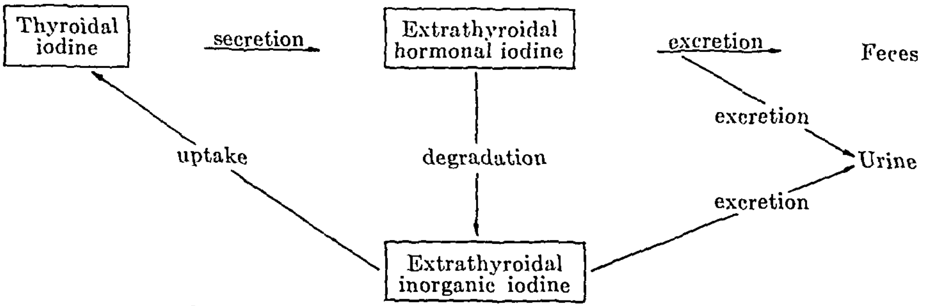
measurements being made 5–7 days after each increase in dose. The initial rates were 7.0 ± 0.1 liters/M^2/hour for the control and 7.2 ± 0.1 liters/M^2/hour for the nephrotics. Exogenous hormones were less effective in increasing the oxygen consumption in nephrotic animals by approximately 30% (Fig. 5).

Effects of Thyroxine on Serum and Urine Constituents in Nephrotics

The effects of thyroidectomy and of *l*-thyroxine replacement therapy were studied in nephrotic and control rats maintained on a constant food intake administered by gavage. The results (Fig. 6 and Table 5) in the two groups are qualitatively similar with regard to changes in body weight, urinary nitrogen and serum cholesterol, but quantitative changes in the last parameter were greater in nephrotic than in control rats. Plasma total protein concentration in both groups rose considerably following thyroidectomy, due to increases in serum globulin, especially α_1; there was a variable effect on the albumin concentration. Administration of 6 μg. of thyroxine daily by subcutaneous injection resulted in incomplete reversion to pre-thyroidectomy levels.

DISCUSSION

A simple model of iodine metabolism may be assumed as the basis for comparison of nephrotic and normal rats:



It was seen that the uptake of inorganic radioiodine and the rate of release of thyroidal radioiodine were increased in nephrotic animals. It was previously calculated that the nephrotics had a small but definite increase in the rate of hormone release (in micrograms per day), of the order of 37–41%, that the urinary excretion of PBI^{131} accounted for 18–23% of the

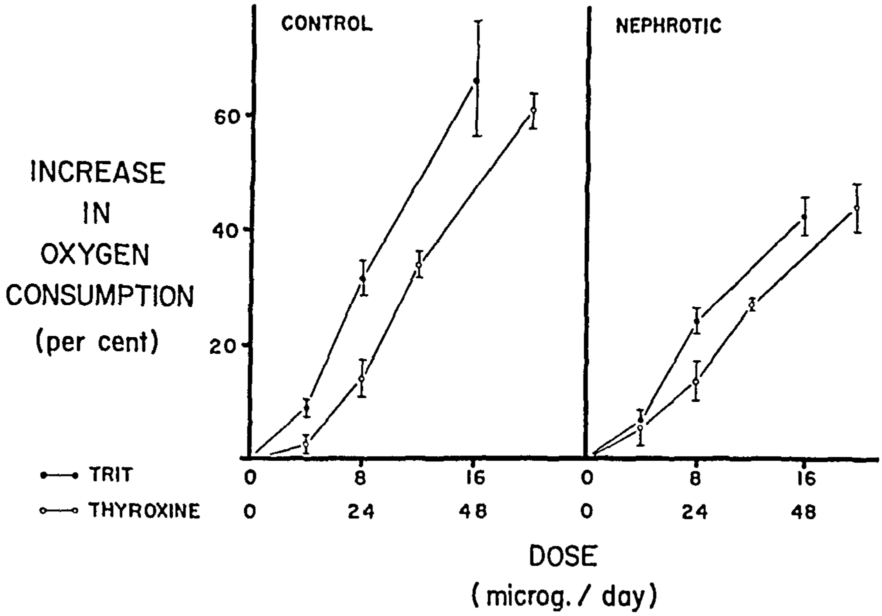


Fig. 5. Effect of *l*-thyroxine and *l*-triiodothyronine on oxygen consumption rates in control and nephrotic rats. Each point is based on 12–45 fifteen-minute periods; vertical bars represent S.E.$_M$.

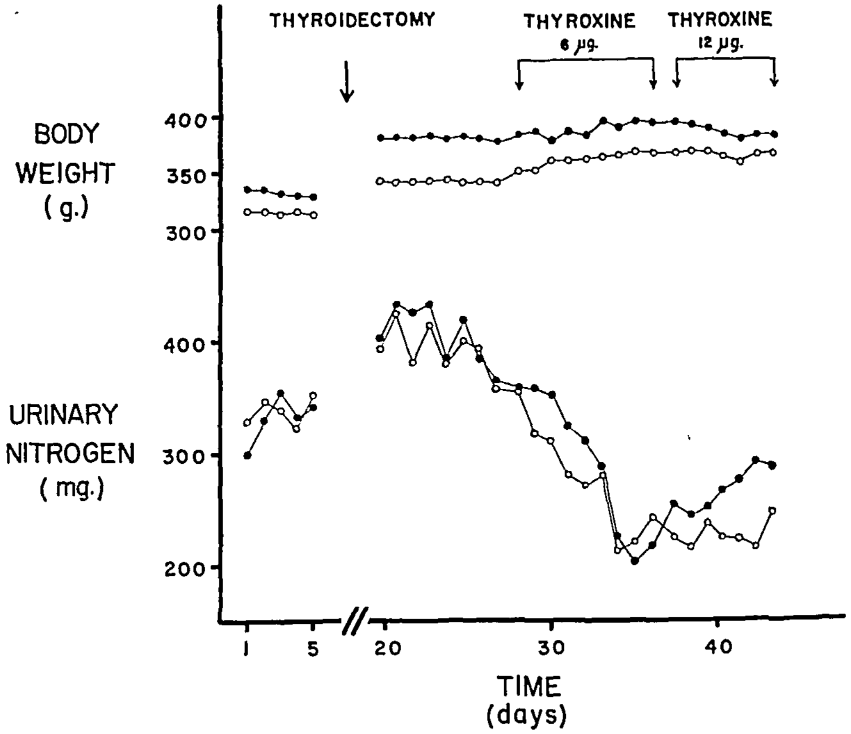


Fig. 6. Effects of thyroidectomy and *l*-thyroxine replacement therapy on body weight and urinary nitrogen excretion in control (•—•) and nephrotic (○—○) rats. Measurements were made daily. Values are means for 6 animals in each group.

TABLE 5. EFFECT OF THYROIDECTOMY AND OF THYROXINE REPLACEMENT ON SERUM CHOLESTEROL AND TOTAL PROTEIN CONCENTRATIONS

	Pre-thyroidectomy	Thyroidectomy	Thyroidectomy +Thyroxine (6 μg./day)
Cholesterol Change (mg./100 ml.)			
Control (6)		+61 ± 9	−25 ± 10
Nephrotic (6)		+100 ± 38	−118 ± 15
Protein (gm./100 ml.)			
Control (6)	7.0	8.9	8.8
Nephrotic (6)	6.2	7.9	8.2

total urinary I^{131}, and that the urinary excretion of PBI^{131} accounted for 19–25% of the radioiodine released from the thyroid gland. The first and last of these figures are, however, minimum and maximum approximations, respectively, since they are based on the net release of I^{131} from the gland. It was unfortunately not feasible to block thyroidal reaccumulation of I^{131} by propylthiouracil, which in the usual blocking dose was lethal for nephrotic rats. Thus the actual release rate was higher and the proportion of secreted PBI^{131} lost in the urine lower than the values calculated. It therefore appears that the increased activity of the thyroid gland could be only partly the result of urinary loss of hormone.

When tube-fed, both normal and nephrotic animals showed a decrease in I^{131} uptake and I^{131} release rates compared to animals fed *ad libitum;* this is in agreement with recent work of Cohn *et al.* (12). It is of interest, however, that the difference between normal and nephrotic animals was still apparent.

The relative specific activity of serum PBI in the nephrotics was ap-

TABLE 6. EFFECT OF THYROIDECTOMY AND THYROXINE REPLACEMENT ON ELECTROPHORETIC PATTERN OF SERUM PROTEINS IN NEPHROSIS

Animal		Alb.	α_1	α_2	α_3	β	γ
Nephrotic	1	2.18	1.88	.89	.31	.73	.63
		2.05	2.28	.59	.89	1.09	1.22
		2.70	1.38	.68	.55	1.17	1.63
	2	1.89	2.16	.54	.41	.67	.41
		1.48	2.74	.84	.68	1.31	1.05
		2.22	2.61	.75	.50	.86	.89
	3	1.06	1.84	.45	.28	.45	.45
		1.17	3.59	.72	.63	.63	.81
		1.55	3.20	1.13	.83	.77	1.03
Control	1	3.50	1.30	.50	.50	1.00	1.10
		3.50	1.50	.50	.60	1.00	1.30
		3.80	1.20	.40	.30	1.40	1.10
	2	3.20	1.30	.40	.40	1.00	1.00
		4.10	1.70	.50	.50	1.80	1.20
		3.50	1.40	.70	.60	1.60	1.50

For each animal values shown are control (top line), thyroidectomy (middle line) and replacement therapy with 6 μg. of *l*-thyroxine daily (bottom line).

proximately twice as great as that of the normals; this is sufficient to explain the increased excretion by the nephrotics of inorganic I^{131} since this was derived from thyroxine degradation.

When labelled thyroxine was injected, the total amounts of I^{131} excreted by the control and nephrotic animals were not significantly different. However, in the latter group a considerable amount of this was lost as thyroxine and consequently not biologically effective.

The degree of such loss varied with the degree of proteinuria. The relationship between the two parameters, as observed in the rat (Figs. 2 and 3), may be explained (a) by a distinct T_M for the thyroxine-binding protein, or (b) by the assumption that the thyroxine-binding globulin is a large or asymmetrical molecule which does not pass the glomerular filter until this is severely damaged, as reflected in proteinuria of 200–300 mg. daily.

The electrophoretic studies were performed by conventional horizontal technique and therefore may suffer from certain artifacts of quantitation in the distribution of the thyroxine (13). However, for comparative purposes the results are valid and indicate, in contrast to the findings of Robbins *et al.* (14), that in the normal rat thyroxine is carried as in man (11, 15, 16), in association with a thyroxine-binding α globulin and with albumin. A similar pattern was obtained in the serum and urine of nephrotic rats.

The oxygen consumption studies indicate a normal rate of oxygen consumption by nephrotic animals. However, responsiveness to thyroxine was decreased by approximately 30%; this might be partly but not entirely attributable to urinary loss of the injected hormone. On the other hand, there was a similar decrease in response to triiodothyronine, yet urinary loss of this material was negligible. The effects of thyroidectomy and thyroxine replacement therapy on nitrogen excretion, serum globulins, serum cholesterol and body weight were qualitatively similar in normal and nephrotic rats, and were similar to those previously described (17, 18, 19). The greater effect of treatment on cholesterolemia in nephrosis is difficult to interpret because of the pre-existing disturbance in cholesterol metabolism.

The findings presented above indicate that in experimental nephrosis a considerable amount of thyroxine may be lost in association with protein in the urine and that there is a decreased responsiveness to administered thyroid hormones which can be attributed only in part to this urinary loss. These abnormalities are apparently compensated by an increase in thyroid activity; the adequacy of compensation is shown by the serum PBI levels and oxygen consumption rates, which under "normal" conditions were equal to those of control animals. The decreased peripheral response to thyroid hormones was apparently unrelated to any gross change in the mechanism of hormone transport.

The present results are compatible with those of Rasmussen (4), obtained in nephrotic patients, indicating that urinary excretion may account for a significant fraction of the thyroxine entering the circulation. If the observed relationship between urinary thyroxine loss and proteinuria applies to the human, disagreement (3, 4) concerning the extent of thyroxine loss may be related to only small differences in degree of proteinuria in the subjects studied.

The relationship of experimental nephrosis to the nephrotic syndrome in humans is far from clear. The gross manifestations are identical, probably because these are consequent on massive proteinuria in both cases. In the present work the major difference between the two states is the apparent inability of the human to compensate for the two abnormalities in thyroxine metabolism, so that the BMR and serum PBI remain low. Rasmussen (4) has postulated that this is due to a degree of hypopituitarism secondary to protein deficiency. That this apparently did not obtain in the nephrotic rat may be a manifestation of differences in the metabolic channeling of available protein rather than of a fundamental difference in thyroid function and iodine metabolism.

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THYROID EPITHELIUM IN TISSUE CULTURES[1]: OBSERVATIONS ON THE MORPHOLOGY AND FUNCTIONAL CAPACITIES OF EMBRYO CHICK THYROIDS

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ABSTRACT

Chick embryo thyroid fragments were cultivated in special tissue culture chambers designed primarily for cytological investigations of the living cell. Employing time-lapse techniques three secretory phenomena were observed. These were associated with (1) cytoplasmic droplets which were equivocally PAS positive and (2) cytoplasmic phase contrast green granules, the localization sites for I^{131}. Chicken whole egg ultrafiltrates stimulated the epithelial outgrowths to a marked degree but this was not apparent for the fibroblastoid stromal elements.

IN 1911 Carrel and Burrows (1) described tissue culture outgrowth of thyroid glands from dogs, cats and guinea pigs predominately as fibroblastoid with occasional epithelial sheet emigrations. Since their presentation four reports (2–4 and 23) have indicated the occurrence of epithelial outgrowths from thyroid tissue cultures but the bulk of analyses (5–21) have been limited to studies of whole gland sections after *in vitro* cultivation. Previous reports indicated that tissue cultures of chick embryo (3) and young adult rabbit (4) epithelium were not functioning. This report introduces data using other tissue culture techniques which demonstrate a functional capacity of chick embryo epithelium.

MATERIALS AND METHODS

(1) *Thyroid source.* Fertile eggs of a hybrid strain (New Hampshire reds×white leghorns) were used as outlined in previous reports (29 and 30) at eight and one-half days and at thirteen days of incubation. Thyroid glands were removed with fine forceps and immediately immersed in *Medium No. 1036* (a variation of Medium No. 858 (31)), trimmed of as much connective tissue as possible and then divided with scalpels.

(2) *Cultivation.* Three or four pieces of divided thyroids were placed on a sterile No. 1 (43×50 mm.) coverslip in a drop of heparinized cockerel plasma. The embryonic thyroid alone produced a firm clotting of the plasma so that additional embryonic extract

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was not necessary. The rubber gaskets and opposing coverslips were then positioned and the multipurpose culture chambers (24–27) closed. Eight to twenty-four chambers were set up in this way for each experiment. Generally, a half or a third of the chambers were selected at random as the control and these were filled with 2 ml. of *Medium No. 1066.* The experimental groups were filled with *Medium No. 1036* which contained varying percentages of chicken whole egg ultrafiltrate (28). Fluids were exchanged after the first two or three days and after five or six days.

(3) *Phase contrast observations and recording.* Each explant was examined with the phase contrast microscope for total growth and epithelial sheet emigration. Epithelial activity was recorded by the time-lapse camera on color and black and white movie film as it was observed through AO-Baker interference and *B&L* phase contrast microscopes (22 and 23).

(4) *PAS staining.* Colloid formation was ascertained by *PAS* staining according to modified techniques of Hotchkiss (32) and others.

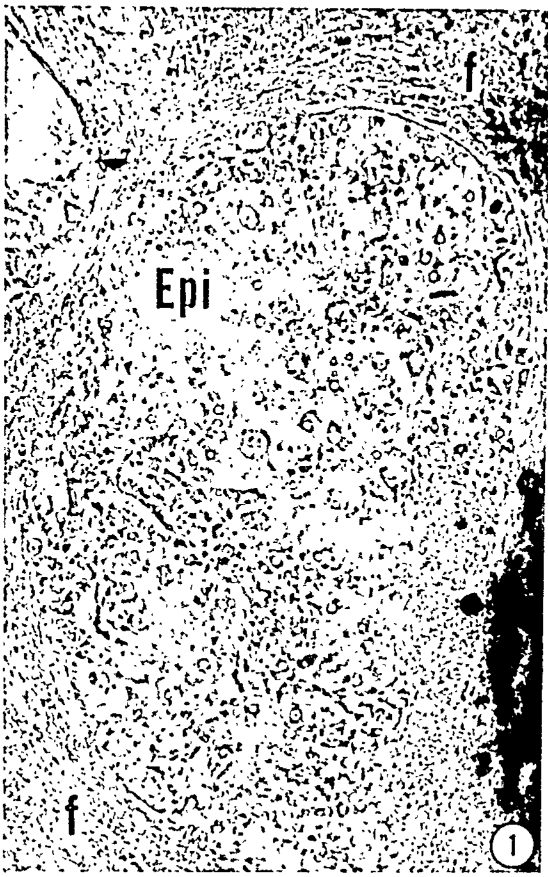
(5) *Radioactive iodine* (I^{131}). Explants cultivated five to six days were exposed for a 24-hour period to nutrients which contained in addition to their control or experimental ingredients I^{131} (1 μ curie/ml.). After this exposure the cultures were fixed in formol, washed in water and covered with stripping film (Kodak) in a manner outlined elsewhere (33).

(6) *Thyroid Stimulating Hormone* (*TSH*). A commercial preparation of the Armour Laboratories (*Thytropar*) was used as the thyroid stimulating hormone in these studies. This lyophilized product was diluted with appropriate amounts of *Medium No. 1033* just prior to its introduction into the chambers so that the cultures received 0.5 unit (U.S.P.) per one ml.

RESULTS

The following general description of development of these thyroid fragments within the culture chambers applies to both the 8½- and 13-day embryo thyroids.

Phase contrast observations. After one day of cultivation a few cells of unclassified origin emigrated around the peripheral edges of the explants. Adjacent to many explants there was a circular or oval lysed area in the clot. In some cases, by the second day, spindle cells emigrated around the peripheral edge of this lysed portion of the clots which had generally enlarged since the first day and syncytia grew on the glass into these clear areas. In other instances, syncytial sheets grew outside of the lysed area and directly upon the surface of the clot. After five or six days of cultivation the epithelial cells had definite membrane borders and the amount of fibroblastoid outgrowth varied widely among the explants (Figs. 1 and 2). Large cytoplasmic droplets occurred within the epithelial cells (Fig. 3), notably in those forming the periphery of the sheets. Generally, these droplets were singular but occasionally cells were noted with many lesser-sized droplets (Fig. 4). Mitotic divisions were rarely observed in these epithelial sheets. The nuclei were regular and small (8–10 μ) and generally had one prominent nucleolus. The cytoplasm contained varying amounts of coarse granules and these too occurred more abundantly in cells at the periphery of the epithelial sheets (Fig. 2). Some of these granules lay adjacent to and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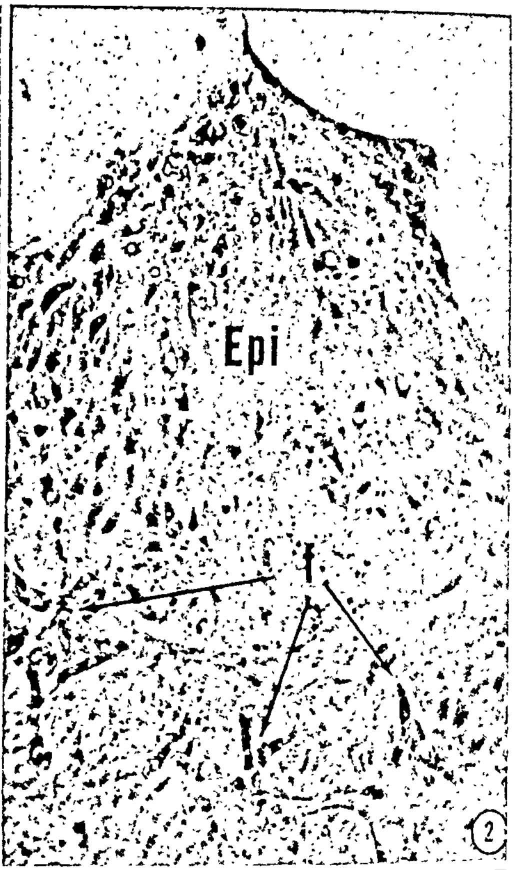
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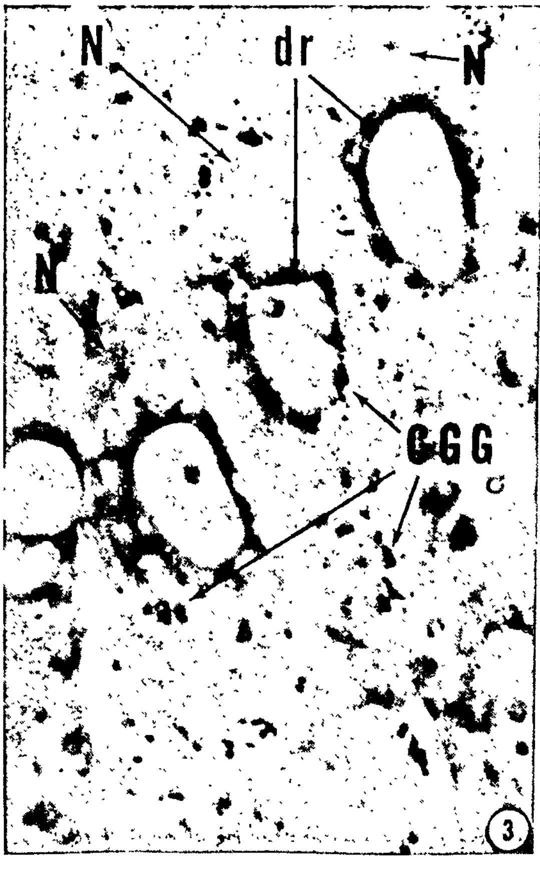
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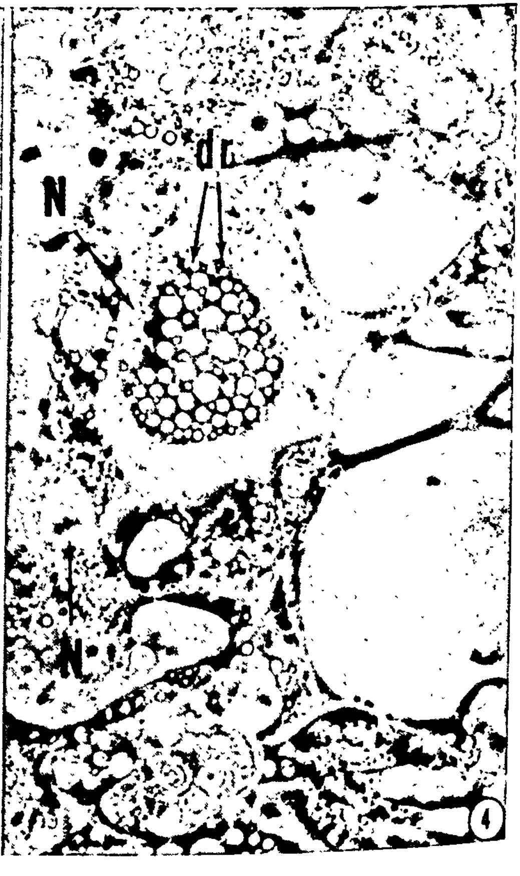
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on the surface of the large cytoplasmic droplets (Fig. 3). At the borders of the epithelial sheets there were points at which the cells were denser, rounder and more granular. Many of these cells also contained the cytoplasmic droplets and several large coarse granules. Careful observation revealed that many of the cytoplasmic granules appeared green regardless of their size when viewed with the phase contrast system (B&L). With bright field optics they were faintly yellow.

Time-lapse motion picture studies. This method of analysis was especially useful for studying epithelial activity. The large cytoplasmic droplets were observed in an effort to determine whether they were involved in a secretory phenomenon. Cells possessing these droplets moved slowly to the edges of the epithelial sheets and there in an un-elicited manner the droplets disappeared as the cells became spheroidal, granular and detached from the epithelium. Some of these detached cells passed into the fluid medium (Figs. 5–9). The fluid of cytoplasmic droplets in other cells was rapidly eliminated (one or two minutes) by a rupture of the droplet wall (Figs. 10–14). A third activity involving a fluid droplet occurred as a rather violent cellular activity which resulted in a protoplasmic flow through a ruptured cell wall. In doing this, the droplet also was lost from the cell (Figs. 15–29).

PAS staining. The explants of cultures subjected to this technique appeared a deep magenta. In some cases the cytoplasmic droplets demonstrated a positive PAS stain but the density of their coloration was light as compared to those of the explants. The phase contrast green granules previously mentioned which are also those cited with reference to radioactive iodine were not involved in this *PAS* staining. Cells which had migrated to the periphery of the epithelial sheets and had rounded as previously described were *PAS* positive even after diastase digestion.

Radioactive iodine (I^{131}). Cultures which were subjected to radioactive iodine *in vitro* and prepared for stripping film analysis demonstrated that

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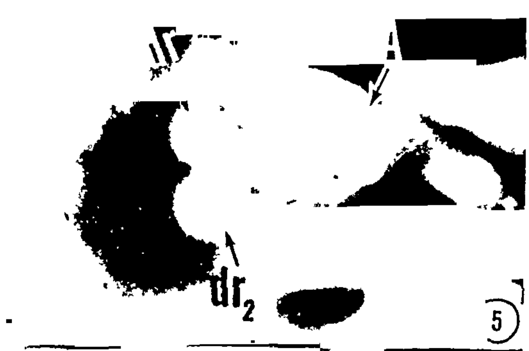
Figs. 1–4. Thirteen-day embryo chick thyroid epithelium growing on glass slides in multipurpose culture chambers for five to seven days as observed through the phase contrast microscope.

Fig. 1. Epithelial sheet (Epi) surrounded by a fibroblastoid stroma (f). ×125.

Fig. 2. Periphery of epithelial sheet with coarse green granules at its limiting edge, cytoplasmic droplets scattered throughout and encroaching fibroblastoid cells (f). ×125.

Fig. 3. Oil immersion (1.8 mm.) view of several epithelial cells which possess large cytoplasmic droplets (dr) and coarse green granules (cgg) scattered throughout the cytoplasm and in close proximity with the cytoplasmic droplets. Note the adjacent location of the nuclei (N) to the three largest cytoplasmic droplets. ×1200.

Fig. 4. Here is a less common occurring multiplicity of cytoplasmic droplets about the nucleus. This formation was seen most commonly in cultures containing serum supplements and was not observed in epithelialization such as demonstrated in Fig. 2. ×550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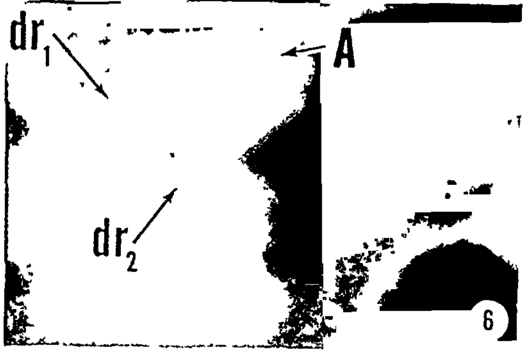
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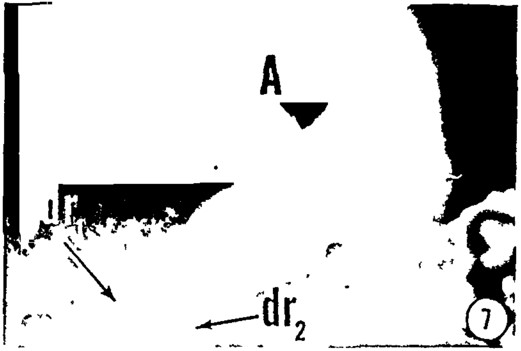
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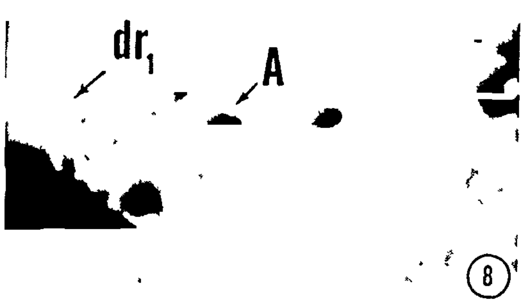
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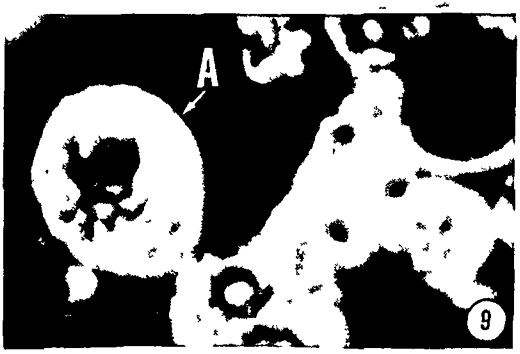
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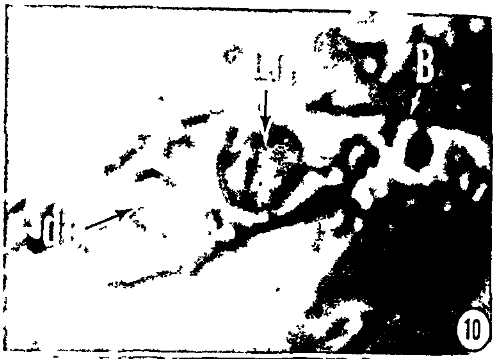
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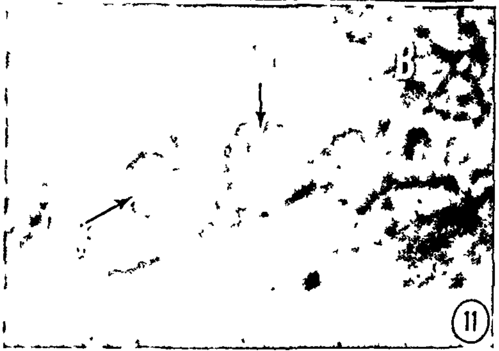
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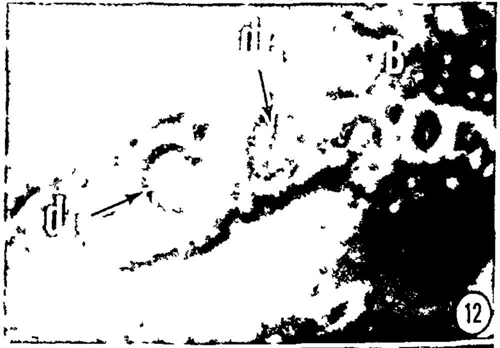
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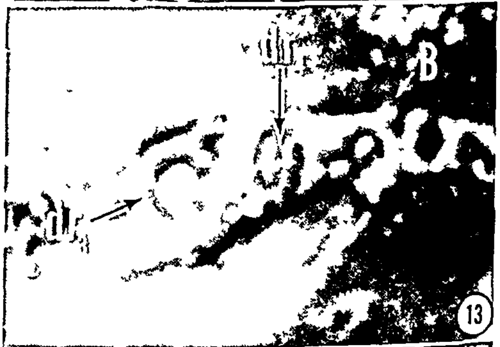
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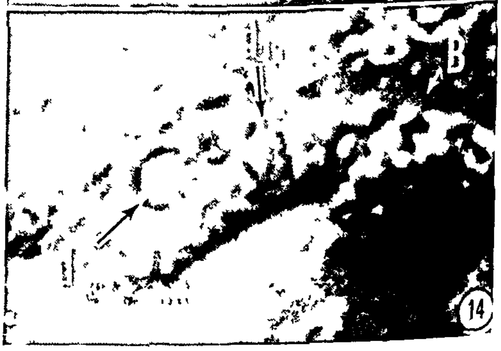
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the coarse cytoplasmic granules (green with phase contrast) were the sites affected by the I^{131}. With the exception of adjacent coarse granules, the cytoplasmic droplets were not involved with the I^{131}. The greatest concentration of radioactivity occurred in cells at the periphery of the epithelial sheets. The adjacent spindle cells occasionally demonstrated an I^{131} concentration; however, explants from supportive tissues which showed only spindle cell outgrowth without clot lysis did not demonstrate this activity. Epithelium cultivated in *Medium No. 1066* with or without the whole egg ultrafiltrate demonstrated I^{131} uptake in a similar way.

Thyroid Stimulating Hormone (TSH). With the time-lapse movie equipment an epithelial cell was observed to develop a perinuclear cytoplasmic droplet after TSH (0.5 U.S.P. units/ml.) was introduced into the nutrient fluid. Following this development, the droplet suddenly disappeared by the method indicated previously (Figs. 10–14) in which the fluid droplet was lost to the nutrient medium by a rupture of its wall.

Growth vs. Chick Whole Egg Ultrafiltrate. Table 1 summarizes the effect on the total growth of explants and their capacity to proliferate epithelial sheets under the aegis of a control (*Medium No. 1066*) nutrient versus one which contained chicken whole egg ultrafiltrate. The top half of Table 1 summarizes the data of the $8\frac{1}{2}$-day chick embryo thyroids cultivated in a control nutrient and in one containing whole egg ultrafiltrate (5%) for 5.5 days. This series comprised a study which involved eight experiments and an analysis of 345 explants. Of the control group, 67% of the explants

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Figs. 5–9. This series was taken from a 16 mm. time-lapse movie sequence and represents an analysis over a four-hour period of an 8.5-day chick-embryo thyroid after five days of cultivation (cell A). The series was originally recorded on Kodachrome "A" film as viewed through the AO-Baker interference microscope. ×1000.

Fig. 5. This cell has two cytoplasmic droplets, dr$_1$ and dr$_2$. The nuclear area is located to the right of these two droplets.

Figs. 6–7. The cell is seen to be moving in a counterclockwise direction and one of the droplets, dr$_2$, was either released or combined with dr$_1$ after Fig. 7.

Fig. 8. Only one fluid droplet is now observable and from observations made of the projected sequence it appeared to be dr$_1$, possibly dr$_1$ plus dr$_2$.

Fig. 9. The cell in this illustration is now a globose mass detached from the epithelium of its origin and ultimately will drift into the fluid nutrient. This type of cell demonstrated the most marked uptake of I^{131}.

Figs. 10–14. This series of an 8.5-day chick embryo thyroid cultivated for five days was similarly taken from a 16 mm. time-lapse movie originally made on Kodachrome "A" film and observed through the AO-Baker interference microscope. The sequence shows a pair of prominent cytoplasmic droplets (dr$_3$ and dr$_4$) contained within cell B. Several smaller fluid droplets are also noted. One droplet (dr$_4$) which is the most central and largest may be followed to rapidly diminish in size. In the movie projection this was visualized as a "popping" of the fluid droplet and rendered the impression that the fluid was suddenly released through the thin cellular wall into the fluid nutrient. The sequence time was four minutes. ×1000.

TABLE 1. GROWTH RESULTS AFTER 5.5 DAYS OF CULTIVATION OF 8.5-DAY CHICK EMBRYO THYROIDS (SUMMARY OF EIGHT EXPERIMENTS)

	Control (1066)			Experimental (1066-WE 5%)		
	(a)	(b)	(c)	(a)	(b)	(c)
Totals	178	119	36	167	128	63
Growth		67%			77%	
Epithelium		20%			38%	

GROWTH RESULTS AFTER 5 DAYS OF CULTIVATION OF 13-DAY CHICK EMBRYO THYROIDS (SUMMARY OF EIGHT EXPERIMENTS)

	Control (1066)			Experimental (1066-WE 5%)		
	(a)	(b)	(c)	(a)	(b)	(c)
Totals	201	180	45	245	232	188
Growth		90%			95%	
Epithelium		22%			77%	

Control (1006). Explants in plasma clots (no EE) and fluid nutrient composed of Medium #1066.

Experimental (1006-WE 5%). Explants in plasma clots (no EE) and fluid nutrient comcomposed of Medium #1066 with whole egg ultrafiltrate (5%).

(a) Total number of explants for given experiment.

(b) Number of explants demonstrating any type of cellular emigration.

(c) Number of explants demonstrating epithelial sheet emigration.

Growth. Percentage of explants with emigrating cells of any type.

Epithelium. Percentage of explants with epithelial sheet emigration.

had outgrowths (from a few to an abundance of emigrating cells) whereas in the experimental group which contained the whole egg ultrafiltrate (5%) 77% of the explants demonstrated this type of outgrowth. In the control group, 20% of the explants had some degree of epithelial emigration whereas in the experimental group 38% of the explants had a similar emigration. This represented a 10% increase for the explants with outgrowths and an 18% increase for the explants with epithelial emigrations in the experimental group. The lower half of Table 1 represents a similar analysis of 13-day chick embryo thyroids after five days of cultivation. Both the control and experimental nutrients were identical to those for

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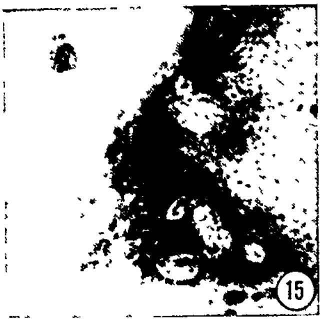
FIGS. 15–29. These pictures of an 8.5-day chick embryo thyroid cultivated for five days were taken from a 16 mm. time-lapse film originally taken on Kodachrome "A." They were observed through the AO-Baker interference microscope over a period of four hours and represent a recorded form of secretory activity. ×600.

FIG. 15. The cytoplasmic area before secretory activity was noted.

FIGS. 16–19. Violent cellular activity (VCA) which preceded the protoplasmic flow onto the glass substrate.

FIGS. 20–22. Here the protoplasmic flow (pf) is indicated to follow the violent cellular activity and occurred at the same position.

FIGS. 23–29. Pictures successively show the emergence of a single large fluid droplet (dr) from the interior of the cytoplasm to its most peripheral portion.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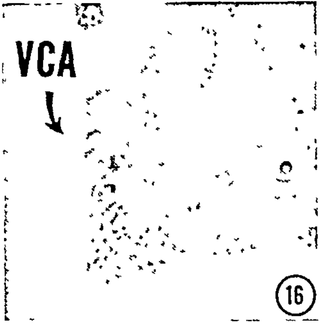

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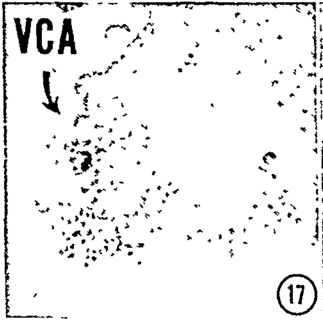

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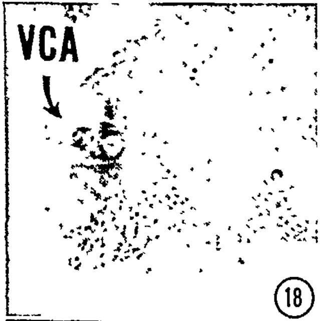

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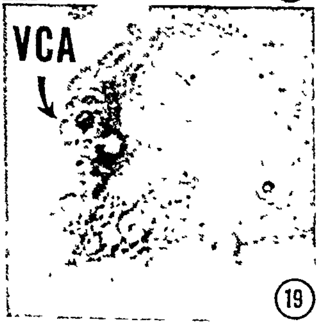

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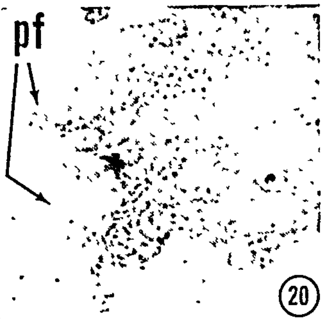

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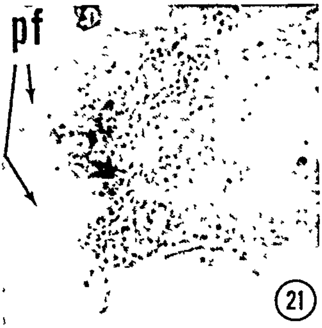

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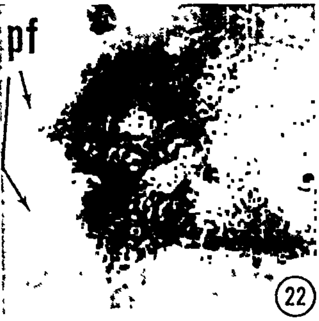

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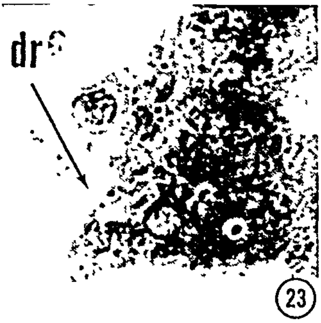

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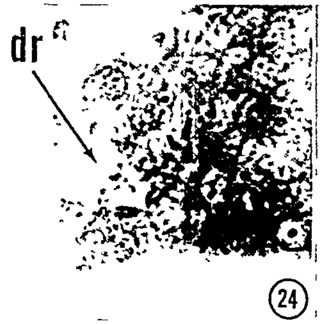

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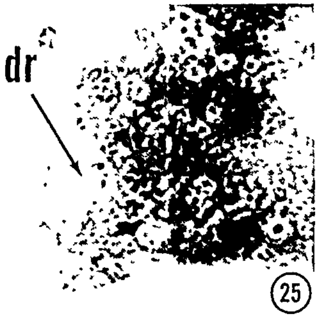

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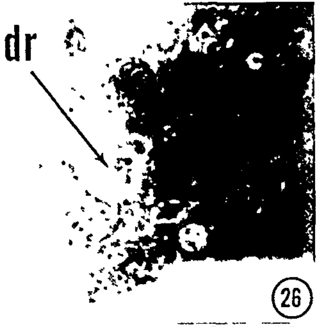

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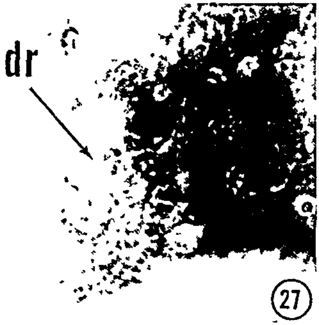

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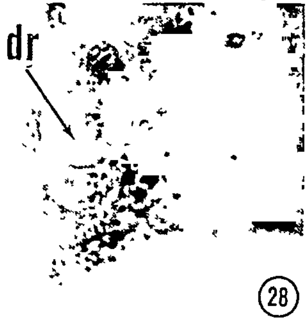

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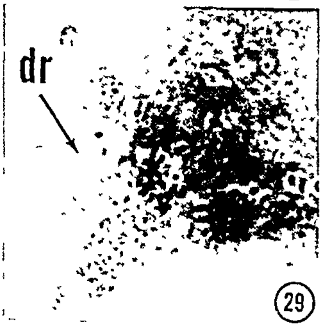

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the 8½-day thyroids. This study involved eight experiments and an analysis of 446 explants. In the control group 90% of the explants demonstrated some type of outgrowth and in the experimental group 95%. In the control group, 22% of the explants demonstrated an epithelial emigration and in the experimental group, 77%. The total outgrowth was then 5% greater in the experimental group which contained the whole egg ultrafiltrate (5%) and the epithelial emigration of the experimental group was 55% greater than the control group. A similar analysis of 13-day chick embryo thyroids in control and experimental groups which contained varying amounts of the whole egg ultrafiltrate (1, 2, 5 and 10%) was carried out in four smaller experiments. The outgrowths in every condition (controls and experimental groups) were in excess of 90% with the exception of the whole egg ultrafiltrate 2% which was recorded at 81%. The epithelial emigration for the control was 33% whereas for the whole egg ultrafiltrate groups it was in excess of 66% with a maximum of 75% for the whole egg ultrafiltrate (5%).

DISCUSSION

Primarily, this study has attempted to show that (1) the epithelial cells which emigrated from embryo chick thyroids were cells which retained at least a part of their functioning capacity and (2) chicken whole egg ultrafiltrate specifically assisted in the reduplication of these epithelial cells by the technique employed. We felt that the first point was a basic consideration which must be proved or disproved before the effect of certain agents incorporated into the nutrient media could be evaluated. If we could not establish that the epithelial emigration consisted of thyroid functioning cells, then any means by which we might stimulate the growth of such an epithelium would have less importance. The fact that the work of Gonzales (3) did not reveal *PAS* positive droplets within the epithelial sheets of chick embryo thyroids cultivated in roller tubes and the fact that Oppenheimer *et al.* (4) were unable to stimulate activity in the rabbit thyroid epithelium after TSH had been incorporated into the nutrient, nor to observe stripping film emulsion reduction after I^{131} had been incorporated into the nutrient did not deter pursuit of this basic information. In the work of Gonzales it seemed quite likely that the droplets of the epithelial sheets were so flat (less than 1 μ) compared to the droplets in the explants themselves that they appeared as a negative *PAS* reaction. When we observed epithelia which contained large cytoplasmic droplets, we considered this possibility and with very critical illumination were able to see these droplets in some cases as a fuchsia of positive *PAS* reaction. Some slides demonstrated this to a greater extent than others.

When 5- and 6-day-old cultures were influenced by nutrients which contained radioactive iodine (I^{131}), fixed and subjected to stripping film photographic techniques, it was noted that the phase contrast green cytoplasmic granules in the epithelial cells were the sites of radioactive uptake. It was felt that the green granules of the living and fixed cultures must have the same optical density and therefore, represented the same organoid. These granules (living or fixed preparations) were prominent in the peripheral cells, particularly in those which had rounded, and, therefore, have taken on a metabolic importance which demands further investigation of this type. Their increasing numbers in the peripherally located cells which are being shed from the sheets point to the functioning capacity of these tissue cultures (34).

In following these epithelial sheets with cinematography we were confronted by three potential means of cellular secretion. All of these methods were observed more than one time so it would be difficult to pass them off only as unusual events. All three phenomena involved the cytoplasmic droplets and, therefore, they were assumed to contain a secretory product. Since the droplets were equivocally *PAS* positive and did not take up the I^{131}, they were probably a colloid source, perhaps a precursor. The luminous-phase-contrast-green granules which did take up the I^{131} were probably associated with thyroxin production.

On several instances the thyroid stimulating hormone *Thytropar* (Armour) was introduced into tissue culture chambers which had been observed for a previous period. Although this was a tedious procedure involving cinematographic and still-camera time-lapse methods it was apparent that the cytoplasmic droplets did increase in numbers during the first twenty-four hours. This was not offered in the *Results* section because occasionally we saw an increase of cytoplasmic droplets occur in cultures which had not received this hormone.

In the analyses which involved thyroid growth in the presence of control (*Medium No. 1066*) and experimental (*Medium No. 1066* plus whole egg ultrafiltrate) nutrients, there appeared to be a consistent increased epithelial stimulation in the 8½-day embryo thyroids as well as a concomitant but lesser increase in the total growth of these explants due to the whole egg ultrafiltrate. The increase did justify further evaluation of another age group (13-day chick embryo thyroid). Within this group we found an extreme epithelial surge which was not paralleled by the total explant outgrowth inasmuch as this was already at a high value (90%). There appeared to be a real need or a mechanism of utilization by this age explant for the whole egg ultrafiltrate as the epithelial response was more than three times greater than in thyroid explants cultivated in its absence. The analysis indicated, too, that even very small amounts of the whole egg ultrafiltrate affected epithelial proliferation. In analyzing the control groups of both 8½- and 13-day chick embryo thyroids the epithelium was often just barely perceptible and in some cases it appeared to be damaged or waning and was not considered to appear as healthy as those cultivated in the whole egg ultrafiltrate. The figures then are misleading with respect to the amounts of epithelium proliferated in the various conditions.

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A DIRECT INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE ADRENAL CORTEX[1]

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ABSTRACT

An *in vitro* comparison of adrenals treated with and without 5-hydroxytryptamine (serotonin) has permitted the demonstration that serotonin has a direct effect on the adrenal cortex. An increase in the secretion of blue tetrazolium (BT) reactive reducing material was elicited by serotonin from the adrenals of rabbits, guinea pig, rat and cow. The response was observable at serotonin levels between 100 to 1000 μg./pair of adrenals. Neither reserpine nor epinephrine had a comparable effect and no alterations in BT-titers were found in kidney and liver slices treated with serotonin. Although serotonin had a similar quantitative effect like ACTH on adrenal BT content, the serotonin influence could be distinguished by the appearance of non-α-ketolic reducing material. The nature of the increased BT material has been partially suggested by its chromatographic and biological behavior. On silica gel columns and toluene-propylene glycol chromatograms, the BT substance(s) migrated similarly to steroids. The material also exhibited sodium retention activity greater than deoxycorticosterone. The lability of the material has prevented identification while the non-α-ketolic reducing compounds have not been investigated at the moment. The possible biological importance of this adrenal reaction to serotonin was discussed.

IN A recent publication Moussatche and Pereiro (1) postulated the liberation of adrenocorticotropin (ACTH) by 5-hydroxytryptamine (serotonin). These investigators obtained a decrease in adrenal ascorbic acid after intravenous injection of serotonin into the intact but not the hypophysectomized rat. It was of interest to us to determine whether serotonin could elicit a direct effect upon the adrenal. By *in vitro* comparison of serotonin-treated and untreated preparations of adrenal tissue, it was possible to assign a stimulating activity of 5-hydroxytryptamine precisely to the adrenal cortex itself. In the presence of serotonin the content of the reducing substances associated with the steroid fraction of the adrenal was increased. Neither reserpine nor epinephrine could bring forth a comparable elaboration of blue tetrazolium reactive material.

METHODS AND EXPERIMENTAL

Adrenals from rabbits, guinea pigs or rats were dissected into quarters or sixths and were incubated for 2 hours in Krebs-Ringer phosphate buffer containing glucose (2, 3). Eight animals of each species were employed so that a representation of each adrenal

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from each animal was present in all the Warburg flasks used in a particular experiment. Adrenal homogenates were also studied and these were prepared by grinding 8 pairs of adrenals in 25 ml. of medium in an all glass homogenizer for approximately 2 min. Debris was removed by filtration through cheesecloth. Adrenal cortical slices from the cow were also investigated.

When glands were relatively large, the incubation flasks contained 30 to 50 mg. (dry wt.) of tissue in 3 ml. of solution. Less available tissue (8 to 15 mg. dry wt.) as in the case of the rat adrenals was incubated in 2 ml. volumes. The compounds to be tested were tipped in from the side arm after the manometers were equilibrated. Astwood's oxycellulose purified ACTH was used as a standard to indicate the capacity of the adrenal to respond and was compared with 5-hydroxytryptamine (as the creatinine sulfate), reserpine and epinephrine. The gaseous phase was always 100% oxygen.

After incubation, the medium and a ½ or 1 ml. rinsing of the flask were transferred to a 5 or 10 ml. glass stoppered tube containing an equal volume of specially purified methylene chloride (2) and shaken. Aliquots of the organic layer from a single extraction were subjected to analysis for reducing material. In the blue tetrazolium test (BT), the sample was dissolved in 0.5 ml. ethanol and 0.25 ml. of 0.018N tetramethylammonium hydroxide was introduced followed by 0.25 ml. of 0.1% blue tetrazolium in ethanol (3). An estimate of non-α-ketolic substances like tocopherols, vitamin K and 5-hydroxyindole derivatives that might be extracted into the methylene chloride was obtained by the ferric chloride-α,α′-dipyridyl test (EE)(3).[2] The sample was dissolved in 1.5 ml. ethanol and alternately mixed with 0.25 ml. of 0.4% α,α′-dipyridyl in absolute ethanol and 0.25 ml. of 0.2% ferric chloride in methanol. Readings at 520 mμ were taken 30 seconds after addition of the ferric chloride. Subtraction of EE values from BT levels would correct the steroid samples for possible non-steroid substances that give a positive BT test.

Attempts at isolation of the reducing material were carried out by incubating larger quantities of rabbit and rat adrenal tissue. Representative portions of each adrenal from 32 rats were incubated with and without serotonin under similar conditions used for the small scale studies. This was repeated using the whole or demedullated adrenals from groups of 12 rabbits. The pooled quartered glands served as their own controls; an initial 1¾ hour incubation contained no serotonin and was followed by an identical interval of time with fresh medium and serotonin. The corresponding methylene chloride extractions were taken down to dryness under vacuum and the residues were chromatographed. Pooled residues from extracts of adrenals from more than 100 rabbits were introduced onto a gm. 5 silica gel column which was eluted consecutively with 40 ml. volumes of benzene, benzene-methylene chloride mixtures, ethyl acetate and methanol. Pooled residues from extracts of adrenals from less than 30 rabbits were subjected to paper chromatography in toluene-propylene glycol or toluene-chloroform-ethylene glycol for 16 to 24 hours (4).

RESULTS

The results of these studies are given in Tables 1 and 2 with all determinations performed in duplicate. Neither creatinine nor sodium sulfate could elicit the response of serotonin creatinine sulfate. In Table 1 may be seen the influence of serotonin on quartered rabbit adrenal. A clear increase in blue tetrazolium reactive material has been elicited. Identical results were obtained with demedullated adrenal preparations. This increment in reducing substances becomes especially significant since the

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[2] Rosenkrantz, H., *Arch. Biochem. & Biophys.*, in press.

TABLE 1. THE INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE CONTENT OF REDUCING MATERIAL IN THE QUARTERED RABBIT ADRENAL*

Experiment	Additive	Corrected BT†	EE	Change
		μg./100 mg. dry weight		%
1	0	76	0	
	ACTH‡	161	0	+110
	1 mg. 5-HTA§	127	97	+ 68
2	0	78	0	
	ACTH	121	0	+ 55
	0.5 mg. 5-HTA	117	27	+ 50
	1.0 mg. 5-HTA	122	53	+ 56
3	0	24	0	
	ACTH	43	0	+ 79
	0.5 mg. 5-HTA	41	28	+ 70
	1.0 mg. 5-HTA	75	48	+212
	1.0 mg. epinephrine	14	0	− 41
	0.5 mg. reserpine‖	20	14	− 16
4	0	45	0	
	1.0 mg. 5-HTA	123	15	+173
	1.0 mg. reserpine	53	5	+ 17

* The rabbits weighed approx. 2000 gm. and both sexes were used.

† EE and BT values are based on serotonin and cortisol standards. Subtraction of the EE titers from the BT level corrects for interference by serotonin metabolites that may have been extracted. Serotonin and α-ketolic steroids give approximately equimolar densities in the BT test.

‡ 8 units of Astwood's ACTH used.

§ 5-HTA = 5-hydroxytryptamine (serotonin) creatinine sulfate.

‖ Added as a suspension.

TABLE 2. THE INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE CONTENT OF REDUCING MATERIAL IN QUARTERED ADRENALS FROM OTHER SPECIES

Species	Additive	Corrected BT*	EE	Change
		μg./100 mg. dry weight		
Guinea pig	0	7	0	
(600 g., males)	ACTH	20	0	+185
	0.5 mg. 5-HTA	9	1	+ 22
	1.0 mg. 5-HTA	23	9	+100
	1.0 mg. reserpine	6	1	− 14
	1.0 mg. epinephrine	6	1	− 14
Rat	0	260	0	
(250 g., females)	ACTH	310	0	+ 19
	1.0 mg. 5-HTA	300	200	+ 15
Rat	0	171	0	
(250 g., males)	ACTH	200	32	+ 17
	1.0 mg. 5-HTA	210	231	+ 22
Rat†	0	200	0	
(250 g., males)	5-HTA	280	120	+ 40
Cow	0	26	0	
(slices)	ACTH	30	0	+ 15
	1.0 mg. 5-HTA	31	5	+ 19
	1.0 mg. epinephrine	21	0	− 19
	1.0 mg. reserpine	22	0	− 15

* EE and BT values are based on serotonin and cortisol standards. Subtraction of the EE titers from the BT level corrects for interference by serotonin metabolites that may have been extracted. Serotonin and α-ketolic steroids give approximately equimolar densities in the BT test.

† Determinations from a large scale incubation.

values of the non-α-ketolic compounds (those that react with ferric chloride-dipyridyl) have been subtracted from the total BT content. A plot of the elaboration of ferric chloride and blue tetrazolium reducing material, respectively, is given in Figure 1 for varying concentrations of serotonin. In the case of the EE curve, EE control levels were subtracted from each point. For the BT content both BT control levels and total EE titers were subtracted at each dose level of serotonin. It can be seen in Figure 1 that after a slight lag period that both EE and BT concentration of the incubation medium increase similarly and plateau at a serotonin level near 1000 µg.

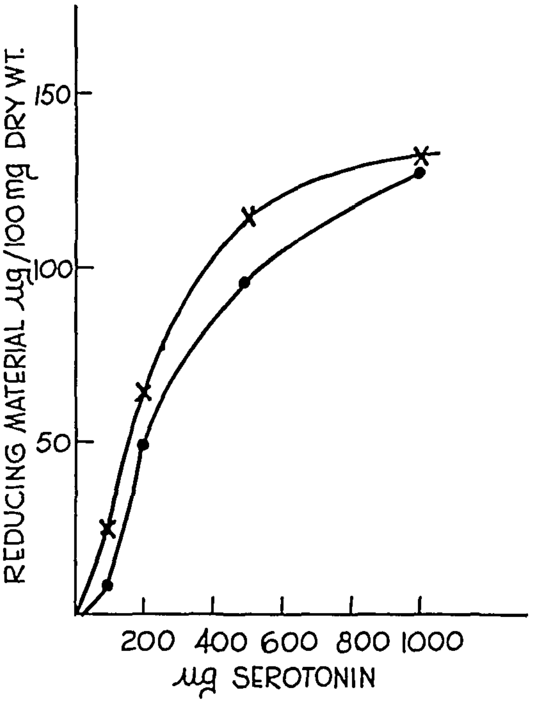

FIG. 1. The elaboration of reducing material from rabbit adrenals induced by serotonin: X——X ferric chloride-dipyridyl reaction. •——• blue tetrazolium levels.

That this is not a non-specific effect of serotonin is demonstrated by the lack of a comparable effect by epinephrine or reserpine (Table 1). The elevation of adrenal BT material is of the same order of magnitude as brought forth by ACTH stimulation. The mean percentage increase due to ACTH was 81 while that of serotonin was 105. Both substances required oxygen and glucose for maximum effect. It is realized that the dosages used for both ACTH and serotonin are much greater than those needed for *in vivo* stimulation. Kidney and liver slices were used as control tissues and serotonin did not alter their BT-titers.

A distinction between the ACTH and serotonin effects can readily be made on the basis of the EE reaction. Little or no material which gave a positive EE test was produced in the presence of ACTH while 5-hydroxytryptamine caused the release of significant quantities of this material.

5-Hydroxytryptamine did not interfere with either the BT or EE analyses since less than 0.5% of it was extracted into the organic solvent.

Examination of Table 2 confirms the direct influence of serotonin on quartered adrenals in other species. The adrenals from guinea pig, rat and cow responded to serotonin with a significant increase in BT material. However, it may be observed that this stimulatory influence was not as profound for the rat and cow adrenal as in the cases of the rabbit and guinea pig adrenals. The EE reducing material also increased and this was true for the cow adrenal too. Although the response of adrenal cortical slices from the cow is poorly discernible, it was felt that a shorter period between obtaining the glands at the slaughter house and performing the experiment would possibly improve the stimulation.

The influence of 5-hydroxytryptamine on adrenal homogenates was inspected and a species difference becomes obvious since rabbit adrenal homogenates responded to ACTH and serotonin while adrenals from guinea pig and rat did not. This would confirm the findings on ACTH and adrenal homogenates reported in a previous paper (3).

In an attempt to elucidate the nature of the BT material formed through the action of serotonin large scale incubations were performed. For both the pooled rat and rabbit adrenals which were not treated with serotonin traces of BT reactive zones could be detected on paper chromatograms. BT reactive zones were more readily detectable on paper chromatograms containing the residue from serotonin treated glands. For both the rat and rabbit the BT positive substances from the control incubations migrated at rates similar to the cortisol-aldosterone-cortisone type of compound and corticosterone, respectively. In a 16-hour chromatogram in the propylene glycol system, corticosterone had migrated approximately 35 to 40 cm. while the unresolved zone of cortisol, cortisone and presumably aldosterone occurred between 1–3 cm. The development of the chromatogram was held to 16 hours so that corticosterone would remain on the chromatogram. Extracts of the serotonin incubation showed no increase in the material migrating at the rate of corticosterone. The cortisol-aldosterone-cortisone zone contained the BT material which had increased in the presence of serotonin. Visual estimation indicated that 5 and 28% of the total BT material determined before chromatography was recovered for the rat and rabbit, respectively. These low recoveries in addition to the sensitivity of the original methylene chloride extracts to temperatures near 50° C suggested that the compound(s) was labile.

The toluene-chloroform-ethylene glycol system gave more definitive results. In this system cortisol migrated 5 to 8 cm. and cortisone 13 to 18 cm. after 16 hours. In both control and serotonin chromatograms, BT reactive zones were detected which corresponded to the mobility of cortisol, but only the serotonin chromatogram contained an intensely reactive BT zone between 9 to 12 cm. Chromatographic results were much less clear for residues containing higher concentrations of lipids. In this situation the BT material that was increased by serotonin migrated over an extended area of the chromatogram with the lipids. Elution of the BT zone gave recovery values below 30%. The acetylation procedure of Mattox *et al.* (5), usually helpful in the isolation of aldosterone, did not yield any products.

It was hoped that larger pools of adrenal incubations would lead to isolation of the BT material. The residues from control and serotonin incubations of adrenal glands from approximately 100 rabbits were respectively chromatographed on silica gel columns. The results of a representative column chromatography are given in Table 3. It can be seen that the BT material elevated by serotonin occurred near the polar end. Approximately 80 to 90% of the BT material could be recovered and the BT material from control incubations concentrated in the methanol fractions whereas the serotonin released reducing substances partitioned between the ethyl acetate and methanol fractions.

TABLE 3. SILICA GEL CHROMATOGRAPHY OF CONTROL AND SEROTONIN RESIDUE FROM RABBIT ADRENAL EXTRACTS

Solvent	Control		Serotonin	
	Eluate wt.	BT*	Eluate wt.	BT*
	mg.	μg.	mg.	μg.
Benzene	59.9	340	16.6	40
Benzene	3.0	40	1.0	10
Benzene	1.1	10	0.6	6
Benzene-methylene				
Benzene chloride 20:1	0.9	12	1.1	4
Benzene chloride 5:1	0.9	10	0.5	14
Benzene chloride 1:1	0.3	11	0.9	10
Methylene chloride	0.5	16	0.6	6
Ethyl acetate	14.4	24	5.2	1100
Methanol	6.3	300	5.1	1050
Methanol	0.4	40	1.0	20
Total recovered from column	88.3	803	32.6	2260
Total added to column	90.2	922	32.6	2897

* The total μg. of BT material per eluate was estimated via a cortisol standard.

Aliquots from the steroid fraction from the silica gel chromatography and corresponding zones from paper chromatography were submitted for electrolyte bioassay.[3] Eight 150 gm. adrenalectomized male rats received an intraperitoneal injection of 45 mg. NaCl and urines were collected for 6 hrs. Simultaneous bioassay was performed on a solvent control, 2 standards of deoxycorticosterone (DOC) and the unknowns. Slopes, test for parallelism and relative potencies were calculated according to accepted procedures. The results are given in Table 4. It was found that 0.037 and 0.094 μg. of the serotonin and control ethyl acetate fractions, respectively, were equal to 1 μg. of DOC. For the methanol fractions 0.051 μg. of the serotonin residue was equal to 1 μg. DOC while the control residue was inactive. These findings indicated that the serotonin ethyl acetate fraction was approximately 3 times as potent as the corresponding control fraction. Since the control methanol fraction did not exhibit any activity in sodium retention, the serotonin methanol fraction was strikingly more active.

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[3] We express our deepest appreciation to Dr. Eugenia Rosemberg for carrying out the bioassays.

TABLE 4. ELECTROLYTE BIOASSAY OF ETHYL ACETATE AND METHANOL FRACTIONS FROM SILICA GEL CHROMATOGRAPHY

Experimental groups	Dose*	Urinary sodium mean ±S.E.	Urinary potassium mean ±S.E.	Potasium/sodium mean ±S.E.
	μg.	mg./100 gm./hr.	mg./100 gm./hr.	
Control		2.44 ±0.28	1.22 ±0.64	0.52 ±0.04
Deoxycorticosterone	6	1.30 ±0.73	0.78 ±0.05	0.60 ±0.02
Deoxycorticosterone	25	0.77 ±0.02	1.09 ±0.04	1.41 ±0.04
Control ethyl acetate	0.2	1.61 ±0.11	1.06 ±0.11	0.66 ±0.01
Control ethyl acetate	1.0	1.09 ±0.27	0.92 ±0.12	0.90 ±0.08
Serotonin ethyl acetate	0.2	1.27 ±0.33	0.78 ±0.14	0.71 ±0.13
Serotonin ethyl acetate	1.0	0.80 ±0.14	0.63 ±0.06	0.72 ±0.12
Control methanol	0.2	1.52 ±0.33	0.77 ±0.09	0.49 ±0.07
Control methanol	1.0	1.85 ±0.51	1.08 ±0.14	0.67 ±0.22
Serotonin methanol	0.2	1.45 ±0.40	0.80 ±0.16	0.61 ±0.08
Serotonin methanol	1.0	0.87 ±0.16	0.77 ±0.10	0.91 ±0.04

* Dose given in terms of BT reactive material.

DISCUSSION

These experiments have demonstrated a direct effect of 5-hydroxytryptamine on the adrenal cortex. The identity of the compound(s) released by the adrenal in the presence of serotonin escapes elucidation at the moment because of its lability. The nature of the non-steroidal reducing material measured by the ferric chloride-dipyridyl reaction also remains unknown. However, since metabolites of 5-hydroxytryptamine would probably retain a potential phenolic ring, they could give the EE reaction. Examination of 5-hydroxyindole acetic acid revealed that this major metabolite of serotonin does give a strong EE test but it is insoluble in methylene chloride and therefore could not account for the EE titers in the present study. Little or no stimulatory activity of 5-hydroxyindole acetic acid on rat adrenal was discerned.

As to the possible explanations for the activity of serotonin on the adrenal gland, two avenues of speculation are available. During hemorrhagic stress serotonin would be released from ruptured platelets. This sudden increment in serotonin titer might directly stimulate the adrenal to release aldosterone-type compounds as a method for altering electrolyte concentrations and combating deranged hemodynamics. It has already been observed that sodium retention occurred in humans receiving serotonin (6) and chloride and water retention has been reported for the rat treated with serotonin (7).

A second possibility approaches an explanation revolving about the concentrations of precursors for corticosteroid synthesis and secretion. Bernheim *et al.* (8) have established that serotonin can function as an antioxidant and can inhibit the formation of lipid peroxides in rat brain homogenate and liver mitochondria. Other catechol and indole amines were much less active. The inhibitory activity of serotonin is particularly pronounced in the presence of ascorbic acid and it is well known that the adrenals are rich in vitamin C. Since cholesterol is a precursor of corticosteroids and can undergo peroxidation, then inhibition of this latter process would afford higher concentrations of precursors for corticosteroid synthesis.

If indeed it is aldosterone that is being released by 5-hydroxytryptamine then a possible common denominator for the varied effects of serotonin on smooth muscle, the kidney, blood pressure and brain activity is suggested. The intense sodium retention and potassium excretion caused by aldosterone could result in an abnormal electrolyte metabolism which might influence kidney function (e.g. reabsorption of chloride and water), osmotic pressure and heart action (e.g. blood pressure) and brain activity (e.g. depolarization of membranes). Since smooth muscle contains an intrinsic network of nerves, shifts in electrolyte concentrations can account for the increased contraction occurring in this tissue also because of polarization effects (7).

Acknowledgment

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PROGESTATIONAL ACTIVITY OF 17-α-METHYL-19-NOR-TESTOSTERONE

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ABSTRACT

The progestational activity of methyloestrenolone and progesterone have been compared in immature female rabbits, using McPhail's method.

Small doses of either drug, when given intramuscularly result in endometrial hyperplasia of equal amount. However, larger doses of progesterone result in greater endometrial hypertrophy than similar doses of methyloestrenolone.

RECENTLY there has been much interest in the biologic effects of newer synthetic steroids including the progestational activity of methyloestrenolone (Orgasteron, Organon). The biological properties of this compound have been studied in women by Ferin (1) and in animals by Overbeek and de Visser (2). The former author has compared the progestational activity of methyloestrenolone to that of progesterone and ethynyltestosterone. Each of these compounds was administered sublingually. Luteoid activity was determined by the glycogen content of the endometrial cells. Ferin (1) found that methyloestrenolone was 150 and 50 times more active when administered sublingually than are progesterone and ethynyltestosterone respectively. Oral methyloestrenolone was found to be 10 times more effective than comparable doses of progesterone given intramuscularly.

The progestational activity of methyloestrenolone is apparently less in rabbits, as determined by the Clauberg test (3). Overbeek and de Visser (2) administered 1.0 mg. of methyloestrenolone *per os* in rabbits. Methyloestrenolone was almost as effective as 1.0 mg. of progesterone when given subcutaneously. These authors also studied the progestational activity in castrated pregnant rabbits and rats. They found that methyloestrenolone had a pronounced progestational property after oral administration. It was possible to maintain pregnancy in castrated animals, at least temporarily, but the fetuses sustained some damage. Toxicity was negligible.

In view of the results of the aforementioned investigators, the author has compared the activity of methyloestrenolone with progesterone when given intramuscularly.

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[1] Fulbright Fellow of Obstetrics and Gynecology at the University of Rochester, School of Medicine and Dentistry, 1957–1958.

TABLE 1. EFFECT OF PROGESTERONE AND METHYLOESTRENOLONE ON ENDOMETRIUM OF IMMATURE RABBITS

Treatment	No. animals	Total dose (mg.)	Transverse endometrial surface area expressed in per cent of values encountered in control animals
No progestative drug	4	—	100
Progesterone	2	0.1	157
	2	0.5	201
	2	0.6	234
	4	2.0	254
Methyloestrenolone	2	0.1	155
	2	0.2	158
	2	0.3	160
	2	1.0	169
	4	2.0	180

MATERIAL AND METHODS

Methyloestrenolone was dissolved in propylene glycol (concentrations of 1:1,000 and 1:10,000). Progesterone was also prepared for intramuscular injection in a similar manner. Twenty-six immature female rabbits,[2] weighing 750–950 gm., were used. All 26 animals were given 25 I.U. of estradiol monobenzoate intramuscularly daily for 6 days. Four animals were used as controls. Ten animals received 0.1 to 2.0 mg. of progesterone. Twelve animals were given 0.1 to 2.0 mg. of methyloestrenolone. Each drug was administered intramuscularly daily for 5 days (see Table 1).

The four control rabbits were given 0.2 ml. of propylene glycol for the same period. On the day after the last injection of methyloestrenolone or progesterone, the rabbits were anesthetized with ether. The uterus of each was removed and fixed in Bouin's fluid for 24 hours. Sections were cut at 10μ across the logitudinal axis of the uterus. The tissue was stained with hematoxylin and eosin. Progestational proliferation of the endometrium was determined by projection of the section on a screen. The total endometrial surface was measured with a planimeter (4, 5). The secretory epithelial area measurement represents a mean value based on many determinations of epithelial surface areas.

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[2] A group of 16 rabbits were studied in the Laboratories of the Dept. of Obstetrics and Gynecology, University of Bologna, Italy, and 10 animals of a second group were studied at the Dept. of Obstetrics and Gynecology, University of Rochester, School of Medicine and Dentistry, Rochester, New York.

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FIGS. 1–7. Cross section of uterus of immature rabbits. Hematoxylin-eosin. ×30.

FIG. 1. 150 I.U. of estradiol monobenzoate I.M. only (control).

FIG. 2. 150. I.U. of estradiol monobenzoate I.M. and 0.1 mg. of methyloestrenolone I.M.

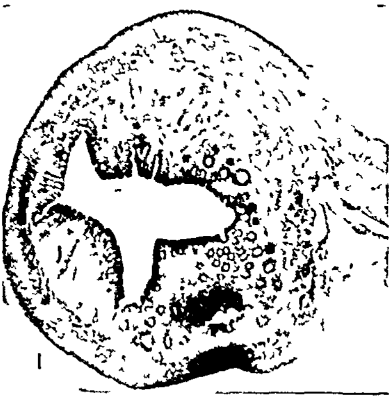
FIG. 3. 150 I.U. of estradiol monobenzoate I.M. and 0.1 mg. of progesterone I.M.

FIG. 4. 150 I.U. of estradiol monobenzoate I.M. and 0.2 mg. of methyloestrenolone I.M.

FIG. 5. 150 I.U. of estradiol monobenzoate I.M. and 0.3 mg. of progesterone I.M.

FIG. 6. 150 I.U. of estradiol monobenzoate I.M. and 2.0 mg. of methyloestrenolone I.M.

FIG. 7. 150 I U. of estradiol monobenzoate I.M. and 2.0 mg. of progesterone I.M.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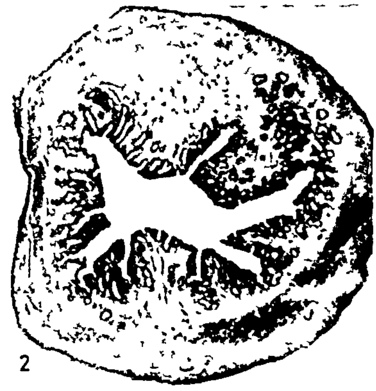
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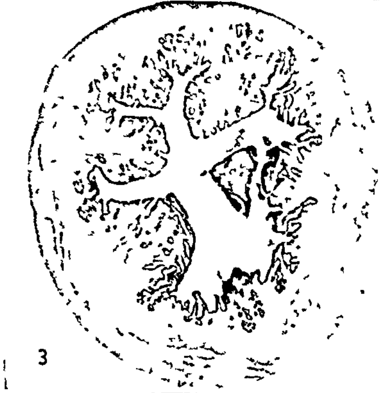
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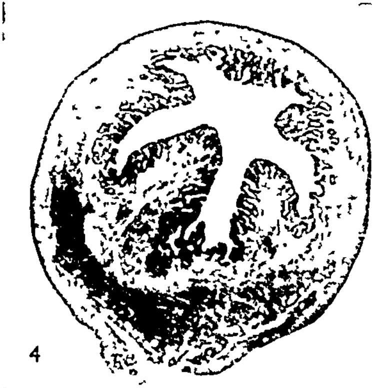
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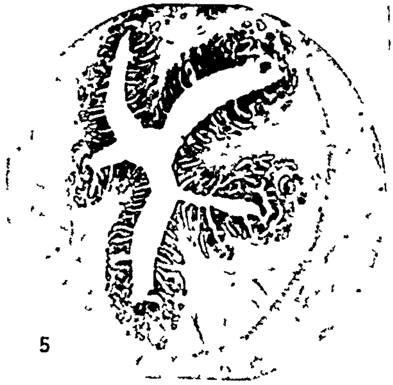
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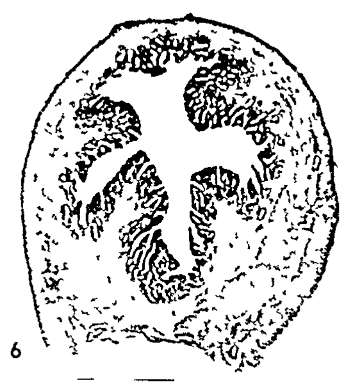
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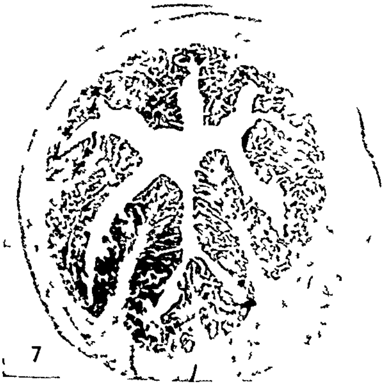
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RESULTS AND DISCUSSION

A dose of 0.1 mg. of methyloestrenolone (given 0.02 mg. daily for 5 days) administered intramuscularly results in endometrial proliferation identical to that of similar doses of progesterone given intramuscularly. Larger doses of progesterone (0.5 to 2.0 mg.) result in much more endometrial hyperplasia than similar doses of methyloestrenolone (see Table 1).

The same results were found in all animals studied. The histological changes induced by each drug are illustrated in Figures 1–7.

The results of the author are thus unlike the findings of Ferin (1) and Overbeek and de Visser (2). The latter workers reported that methyloestrenolone had more progestational effect on the uterus than progesterone, when these drugs were administered orally. The author's results demonstrate that the progestational activity of methyloestrenolone and progesterone, when administered intramuscularly, are similar.

Therefore the comparative progestational activity of methyloestrenolone or progesterone is apparently dependent upon the route of administration as well as the test animal used.

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EFFECT OF THE INJECTION MEDIUM UPON THE BIOLOGICAL RESPONSE TO PARATHYROID HORMONE[1]

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ABSTRACT

Male Wistar rats weighing approximately 115–120 gm. were parathyroidectomized and maintained on a house diet. Four days later, after an initial blood sample was obtained, they received a known amount of parathormone A (purified hydrochloric acid gland extracts) or parathormone B (purified acetic acid extracts) either suspended or dissolved in one of several injection media. They were bled again three, six, nine, twelve, fifteen, eighteen, or twenty-four hours later. The concentration of calcium in the initial and final blood samples was determined, and the results plotted as increase in plasma calcium concentration versus time. It was found that 1) the duration of the biological response was dependent upon the injection medium, being longest when the hormone was given as a suspension in oil and shortest when given in isotonic saline; 2) the magnitude of the response, following the injection of PTH-B was dependent upon the injection medium, this effect being apparent as early as three hours after the test injection; and 3) N-ethyl maleimide in concentrations of 1×10^{-3} *M* or cysteine hydrochloride in concentrations of 1×10^{-1} increased both the magnitude and duration of the response to PTH-B given in isotonic saline. The implications of these findings are discussed.

IN A previous communication, we reported upon an alteration in the duration of action of parathyroid hormone after partial purification (1). This phenomenon was noted with partially purified hydrochloric acid extracts of bovine parathyroid glands having a potency of 75–100 U.S.P. units/mg. dry weight. As was expected, a similar phenomenon was observed upon purification of acetic acid extracts by the procedure previously described (2). Because this phenomenon is of obvious practical importance in the proper assessment of the potency of various parathormone preparations, it was decided to study it in more detail. The results of this study form the basis of the present report.

As has been pointed out (2, 3), the active principle in acetic acid extracts differs in certain regards from that in hydrochloric acid extracts of bovine parathyroid glands. In keeping with the terminology previously proposed (3), these principles will be called parathormone B (PTH-B) and para-

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thormone A (PTH-A), respectively, and these designations will be used throughout this paper.

MATERIAL AND METHODS

Male Wistar rats (CNF strain from Carworth Farms, N. Y.) weighing approximately 115–120 gm. were employed for all experiments. They were maintained on a normal house diet. Four days after parathyroidectomy, they were separated into groups of eight and used for the assays. Parathyroidectomy, anesthesia, heart puncture, and calcium determinations were performed as previously described (3). The animals were allowed free access to water but no food during the course of the experiments.

To test the effect of the various injection media upon the biological response, 0.25 mg. of PTH-B, prepared as previously described (2), or 0.50 mg. of PTH-A, prepared as previously described (3), was taken up in 0.5 ml. of the medium to be tested. After an initial blood sample was obtained by cardiac puncture, the hormone was administered subcutaneously in the right axillary region. A seocnd blood sample was collected from each rat of separated groups of 6 to 8 animals at intervals of 3, 6, 9, 12, 18, or occasionally 24, hours after the test injection. Usually 70 to 80 animals were employed in a given experiment. In some cases, the same group of six or eight rats was bled at 6 and then again at 18 hours, or 9 and 24 hours. Each set of experiments with a given injection medium was repeated at least once. At least one group in each series was given 0.25 mg. of PTH-B in 0.5 ml. of oil, and bled at 6 hours, to serve as a standard of comparison for successive series of experiments, such a procedure being the basis for the assay method previously described (3). The results from group to group were quite uniform after administration of the 0.25 mg. of PTH-B in oil. When given in arachis or sesame oil and assayed by the six-hour method previously described (3), the PTH-B preparations had an activity of 180–200 U.S.P. units/mg. dry weight, and the PTH-A 75–100 U.S.P. units/mg. dry weight. The results have been plotted as the increase in concentration of the plasma calcium versus time after injection. The results obtained with animals in which the initial calcium value was greater than 8.5 mg./100 ml. were not included because these animals were considered to have been only partially parathyroidectomized. The usual initial concentrations of calcium ranged from 6.0–8.5, and only three to four animals in each series of sixty to seventy had values greater than this.

The various solutions employed all were prepared upon the day of the assay, using "deionized" water and reagent grade compounds. The sesame or arachis oil was stored at 2° C and small aliquots were melted as needed. The oil was poured on to the dry hormone power and the mixture shaken vigorously to obtain a fine suspension. For the experiments with heparin the parathormone was taken up in saline or buffer and a drop of two of heparin added from a clean syringe. Upon the addition of the heparin a fine white cloudy precipitate formed and remained suspended in the solution. The gelatin employed was Type B "Pharmagel" gelatin of purest grade obtained from Knox Gelatin Co., Camden, N. J. An approximately 16% solution of this material was prepared by bringing 100 ml. of a pH 4.0 acetate buffer to a boil, removing it from the flame, and then adding 16 gm. of gelatin powder with constant stirring. After the solution had cooled to 40–50° C, 100 mg. of phenol were added and the material stored at 37° C, at which temperature the mixture was a highly viscous solution. None of the gelatin solutions were kept for more than one week, and were usually prepared the day before expected use. The N-ethyl maleimide and *l*-cysteine hydrochloride were obtained from Mann Research Laboratories, Inc., New York, N. Y. The human plasma, from a normal volunteer, was collected and stored with sterile precautions. The anticoagulant employed was heparin. Statistical analyses were carried out by standard methods (4).

RESULTS

In Figure 1 are shown the results obtained when the same preparation of PTH-A was taken up in saline, heparin solution or arachis oil, and injected into separate groups of animals. Each animal received 0.5 mg. of PTH-A in 0.5 ml. of the injection medium. Each point on the chart represents the mean response of 16 to 19 animals. Heparin or oil seem to be equally effective in prolonging the response. It is noteworthy that the response seen six hours after test injection is more or less the same regardless of the injection medium. This being the case, it is apparent that with a six hour assay procedure and these preparations an adequate assessment of biological potency can be obtained using a variety of injection media. This is not the case, however, with the more highly purified PTH-B preparations as can be seen in Figures 2 and 3. In Figure 2 are plotted the responses observed when preparations of PTH-B (0.25 mg. per rat) were injected in oil or a pH 7.4 sodium bicarbonate buffer. Each point represents the mean response of 17–24 animals, and the vertical lines represent the standard errors. The most striking finding, other than the change in duration of action, is the observation that even as early as three hours after the injection there is a significantly greater response when the PTH-B is administered in oil rather than a neutral bicarbonate buffer. Furthermore, the results indicated in Figure 3 illustrate that a graded range of response is obtained depending upon the injection medium employed and the interval of time elapsed before measuring the response. Here again each point represents the mean response of 11–36 individual animals. The standard errors have been omitted for sake of clarity, but were similar

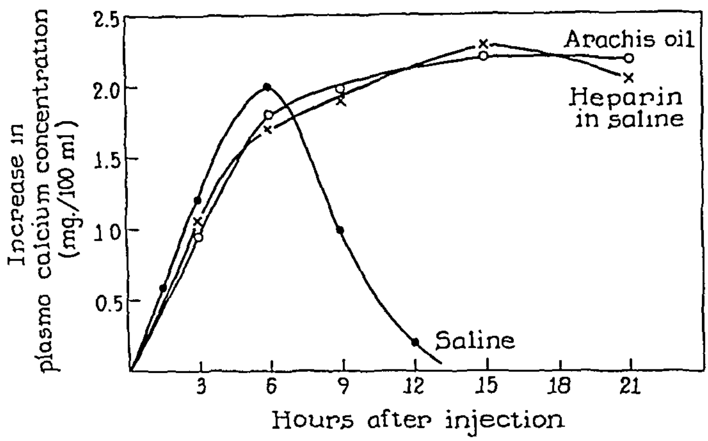

FIG. 1. The change in concentration of calcium in plasma of parathyroidectomized rats after the subcutaneous injection of 0.5 mg. of PTH-A in 0.5 ml. of heparin (X—X), oil (O—O) and isotonic saline (●—●).

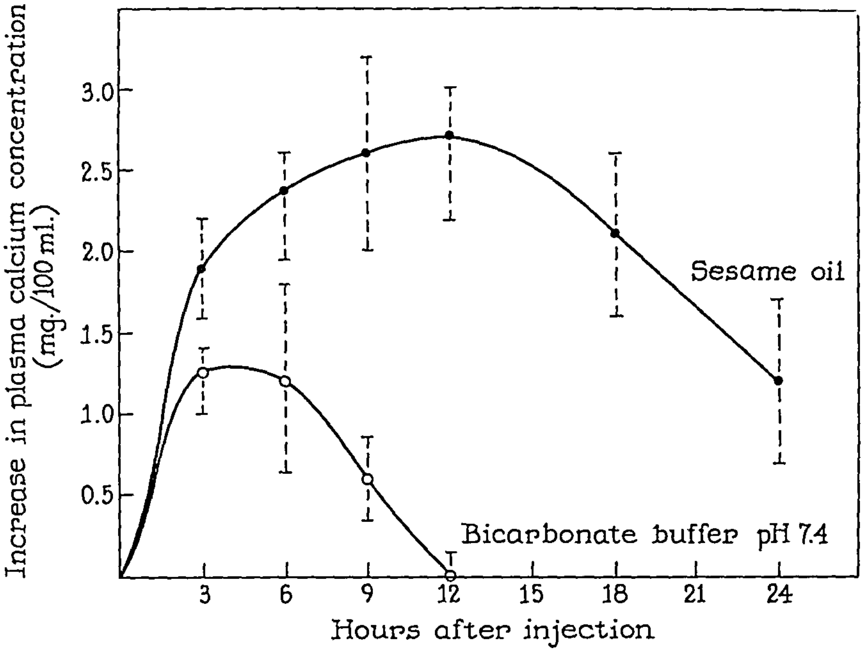

FIG. 2. The change in concentration of calcium in plasma of parathyroidectomized rats after the subcutaneous injection of 0.25 mg. of PTH-B in sesame oil (●—●), and a 0.1 *M* sodium bicarbonate buffer, pH 7.4 (○—○).

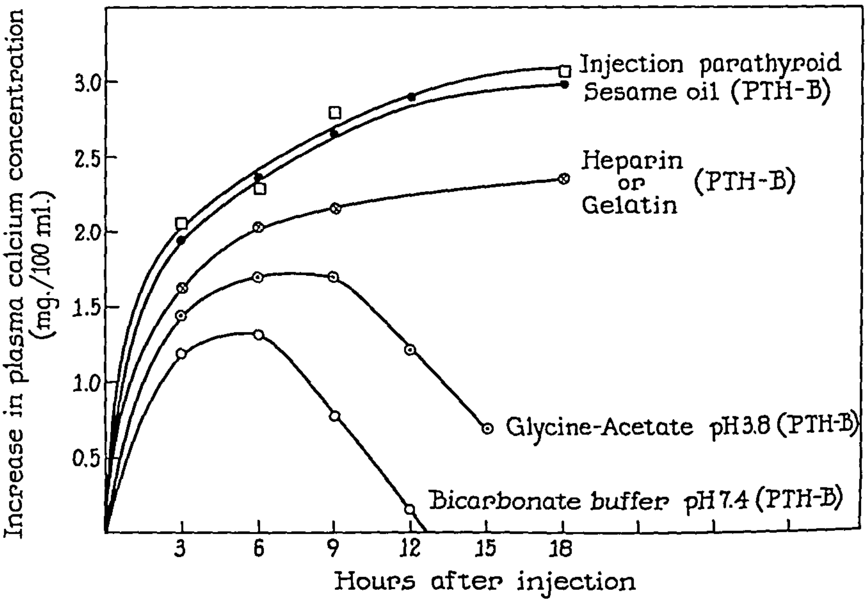

FIG. 3. The increase in concentration of calcium in plasma of parathyroidectomized rats following the subcutaneous injection of 0.25 mg of PTH-B in oil (●—●), heparin, gelatin solutions, or plasma (⊗—⊗), 0.01 *M* glycine acetate buffer, pH 3.8 (⊙—⊙), and 0.1 *M* sodium bicarbonate, pH 7.4 (○—○). The upper curve (□—□) represents the response obtained with 50 U.S.P. units of commercial Injection Parathyroid Hormone (Eli Lilly Company), or appropriate amounts of crude acid extracts of bovine glands.

to those shown in Figure 2. The response to 50 U. of Injection Parathyroid (Eli Lilly) has been included for comparison. A similar response is obtained when an appropriate amount of a crude acetic acid extract of parathyroid glands is injected in saline or oil. In these experiments (Fig. 3), in contradistinction to those with PTH-A (see Fig. 1), the response when the PTH-B was given in oil, rather than with heparin or gelatin, was significantly greater at nine or more hours after injection. The results at six hours, when analyzed statistically (4), were probably significantly different ($P > 0.01 < 0.05$). The response observed at 6 hours when the PTH-B is given in a 0.01 *M* glycine acetate buffer (pH 3.8) is significantly greater than when administered in a neutral bicarbonate buffer ($P < 0.01$).

A single set of experiments was carried out using sterile human plasma as the injection medium. The results obtained were similar to those obtained with the gelatin solutions and have not been plotted. It seemed possible that the heparin in the plasma was an important factor, so plasma obtained with a balanced oxalate solution was also tested during a routine six hour assay. The results were similar to those seen with the heparinized plasma.

Perhaps the most striking results are those shown in Figure 4. By adding 10^{-3} *M* N-ethyl maleimide or 10^{-1} *M* cysteine hydrochloride to saline both the magnitude and duration of the response to PTH-B is increased. The addition of 10^{-1} *M* cysteine hydrochloride to Injection Parathyroid (Eli Lilly) did not change the magnitude or duration of the biological response.

DISCUSSION

The significantly greater response obtained, even as early as three hours after injection, when PTH-B is given in oil rather than in a neutral bi-

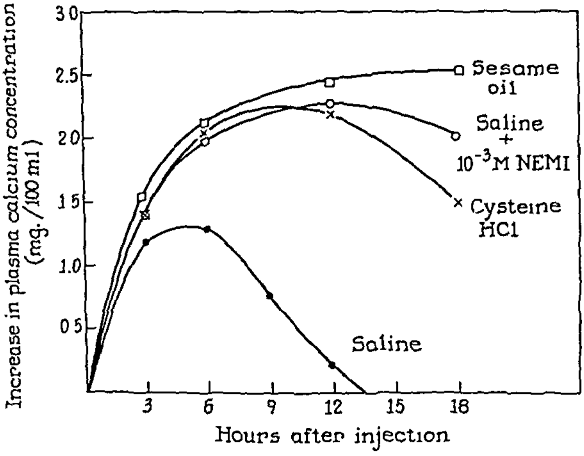

FIG. 4. The alteration in the response to the subcutaneous injection of 0.25 mg. of PTH-B produced by the addition of 10^{-3} *M* N-ethyl maleimide (○—○), or 10^{-1} *M* cysteine hydrochloride (×—×) to isotonic saline (●—●). The response obtained when this particular preparation was injected in sesame oil (□—□) is included for comparison.

carbonate buffer (Fig. 2) suggests that when given in the neutral buffer the PTH-B is partially inactivated at the site of injection. That this interpretation is more likely correct than the assumption that just a more rapid rate of absorption and excretion occurs is suggested by the results obtained with the addition of NEMI and cysteine hydrochloride (Fig. 4), as well as those obtained with glycine acetate buffer (Fig. 3). These results are in keeping with observations (unpublished) that PTH-B is unstable *in vitro* in neutral buffer, is stabilized by the addition of NEMI to these buffers, and is stable in glycine acetate and cysteine hydrochloride buffers.

Initially, it was thought that the effects of NEMI and cysteine hydrochloride could be explained by their known ability to prevent disulfide interchange (5). Further work (unpublished) has shown, however, that PTH-B has neither free SH groups nor contains cystine. Furthermore, it has been shown recently that parathyroid hormone loses biological activity when oxidized with hydrogen peroxide, and that subsequent reduction with cysteine hydrochloride results in the restoration of biological activity (6). In view of these results, it would appear that cysteine hydrochloride and NEMI exert their effect by inhibiting this oxidative inactivation of parathyroid hormone probably at the injection site. It seems possible that these two agents may also prevent the inactivation of the hormone at other sites since they both prolong the duration of action of the hormone. Studies are being undertaken to determine if such a synergistic effect exists.

The difference in the overall response to PTH-B when given in heparin or gelatin rather than oil (Fig. 3) may well be explained by a more rapid rate of absorption from the site of injection, and therefore a less sustained concentration of circulating hormone.

The results recorded in Figure 3 point out the difficulty encountered in assessing the biological potency of purified parathormone preparations; for, depending on the time period of assay used and the injection medium employed, one can find a potency of from 0–200 U.S.P. units/mg. of the same preparation. To date, the most reliable results have been obtained with oil as the vehicle for injection. When oil is employed the more purified preparations form a satisfactory, fine, even suspension, but the crude preparations do not disperse readily.

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IN VITRO AND *IN VIVO* METABOLISM OF PREDNISOLONE: STUDIES CONCERNING ITS BIOLOGICAL EFFECTIVENESS

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ABSTRACT

Metabolism of prednisolone by rat liver microsome-supernatant fractions has been reinvestigated. It has been found that both C-20 and Δ^4-3 ketone reduction of prednisolone is considerably slower than rates of reduction of these substituents on the hydrocortisone molecule. Comparisons of glycogenic activity also resulted in collection of data which suggest that prednisolone persists for a longer period in tissues or body fluids of fasted adrenalectomized rats. When equal amounts of either hydrocortisone or prednisolone were administered intraperitoneally to fasted adrenalectomized rats, it was found that forty-two times as much unaltered prednisoline appeared in the urine following a 48-hour collection period. The significance of these results in relation to increased biological efficacy of the synthetic steroid is discussed.

RECENT reports (1) suggest that rat liver slices do not metabolize prednisolone, 2-methylhydrocortisone, 9α-fluorohydrocortisone and 2-methyl-9α-fluorohydrocortisone at significantly slower rates than hydrocortisone. These results are in conflict with results reported from this laboratory (2) in which microsome-supernatant fractions of rat liver were employed. These results are also in conflict with studies reported by Schriefers (3) in which rat liver slices were used. In addition, Tompkins (4, 5) and Liddle (6) have reported that 2-methylhydrocortisone and prednisolone are metabolized more slowly than hydrocortisone by rat liver enzyme systems. Todd and Hechter (7) have demonstrated a slower rate of 9α-fluorohydrocortisone metabolism by liver paste and liver slices. *In vitro* studies, reported by Rongone (8), clearly indicate that in a bovine serum albumen preparation, in which a TPN-2H-generating system was supplied, the metabolism of both prednisolone and 9α-fluorohydrocortisone was significantly slower than that of hydrocortisone.

Slaunwhite, *et al.* (9) have shown that administration of prednisolone to human subjects results in excretion of more unaltered steroid in urine than that following administration of hydrocortisone. Vermeulen (10) and Gray (11) have clearly shown that considerable amounts of prednisolone appear in urine of patients treated with this hormone. These latter observations, coupled with those of Ely (12), which suggest that the biological half-life of prednisolone in human subjects is considerably longer than that of the

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parent hormone, tend to indicate that metabolism of prednisolone is significantly reduced.

Collins *et al.* (13), as well as Silber and Morgan (14) have shown that the biological half-life of prednisolone is longer than that of hydrocortisone following oral or intravenous administration to experimental animals (dogs). Glenn *et al.* (2) have shown not only an impeded rate of metabolism of 2-methyl-9α-fluorohydrocortisone by rat liver enzyme systems, but have also shown that intravenous administration of this compound to fasted adrenalectomized rats results in a markedly prolonged biological response —results which suggest that 2-methyl-9α-fluorohydrocortisone persists longer in tissues or body fluids. Critical analysis of the different types of data alluded to has fostered the concept (2) that slower rates of liver inactivation may partially explain the enhanced biological potency of certain synthetic steroids.

The following report is concerned with *in vitro* and *in vivo* studies concerning not only metabolism of one of the representative steroids under discussion (prednisolone), but also concerns itself with analysis of additional biological data which also tend to shed light on this subject.

METHODS

Methods for determination of steroid metabolism rates by isolated microsome-supernatant fractions of rat liver have been described in detail (2). Ability of steroids to induce liver glycogen deposition in fasted adrenalectomized rats was determined by previously described methods (15).

For urinary excretion studies, prednisolone and hydrocortisone were suspended in carboxymethylcellulose (5 mg./0.5 cc.) and injected intraperitoneally to fasted adrenalectomized rats. Animals were placed in metabolism cages; urine samples collected for 48 hours, pooled and subsequently extracted with five times volume of 20% ethyl acetate:chloroform. Solvent phases were extracted two times with 1/10 volume of 0.1N NaOH, 0.1N HCl, followed by distilled water extraction. Solvent phases were dried and, since dry residues appeared to be relatively free from extraneous material, total residues were dissolved in $CHCl_3$, transferred to paper strips, and chromatographed by the Bush (16) system. Steroids were identified by comparison of Rf values with standards, absorption spectrum in the ultraviolet, the Porter-Silber (17) method for detection of the dihydroxyacetone side chain, and H_2SO_4 chromogens in both ultraviolet and visible spectra. Determination of amounts of steroid present in eluates from paper strips was accomplished by ultraviolet and Porter-Silber methods of analysis.

RESULTS

When equimolar amounts (0.1 cc. of an 0.01 *M* solution) of hydrocortisone and prednisolone were incubated with isolated microsome-supernatant fractions of rat liver, it became readily apparent that both the Δ^4-3 ketone and C-20 ketone positions of the latter were reduced at considerably slower rates than the former (Fig. 1).

If a slower rate of liver inactivation of prednisolone is partially responsible for its increased glycogenic activity, one would expect a more prolonged

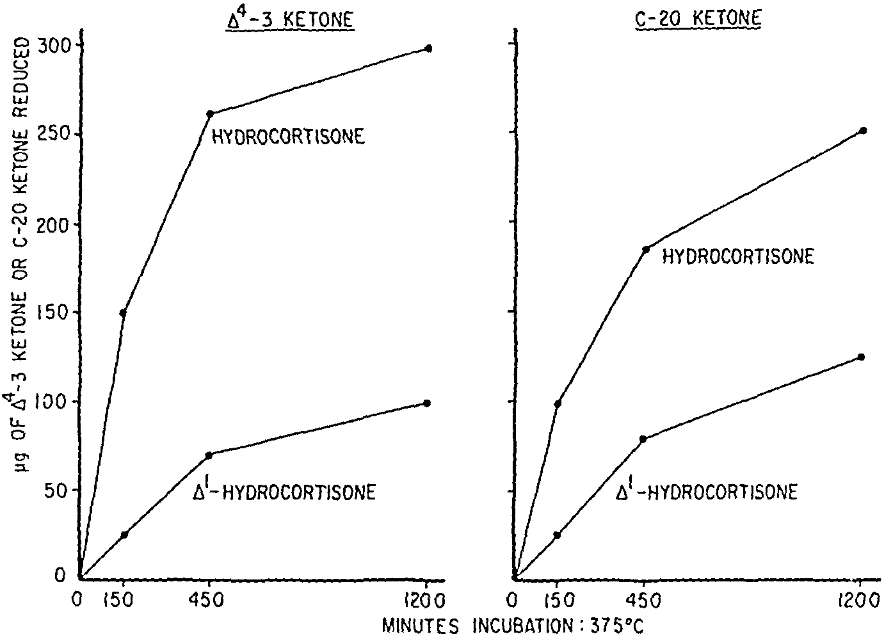

FIG. 1. Rate of Δ^4-3 ketone and C-20 ketone reduction of hydrocortisone and Δ^1-hydrocortisone (prednisolone) by isolated microsome-supernatant fractions of rat liver. (Each point: mean of duplicate determinations on duplicate samples.)

biological response following intravenous administration. When 1.0 mg. of prednisolone and 1.0 mg. of hydrocortisone (in 0.1 cc. EtOH) were administered intravenously (tail vein) to fasted adrenalectomized rats, results indicated that liver glycogen deposition response to prednisolone was more prolonged than response to hydrocortisone (Fig. 2).

Studies reported thus far indicate that prednisolone is metabolized more slowly by rat liver enzyme systems and, in keeping with this observation, a more prolonged biological response is obtained following its intravenous administration.

If metabolism of prednisolone is actually decreased in the fasted adrenalectomized rat, one would expect more of the "free," unmetabolized steroid to appear in urine of animals treated with this material. In order to be assured that both hydrocortisone and prednisolone would be equally exposed to liver inactivation, and in order to assure relatively complete absorption of both compounds, they were administered intraperitoneally. Hydrocortisone and prednisolone (10 mg./rat) were administered to two groups of twelve rats per group. Urine was collected over a 48-hour period, pooled, and analyzed according to methods described previously.

Following intraperitoneal administration of 120 mg. of hydrocortisone to twelve fasted adrenalectomized rats, 188 μg. of hydrocortisone were recovered in pooled urine samples. Following intraperitoneal administration of 120 mg. of prednisolone, 7,860 μg. of free, unmetabolized substrate appeared in urine extracts. That is, forty-two times as much prednisolone appeared in the urine as did hydrocortisone. Since the amount of "free," nonmetabolizable steroid appearing in urine of animals treated with this hormone is probably a relative measure of reductive and conjugating mechanisms in liver, the conclusion seems warranted that liver of the fasted adrenalectomized rat metabolizes the steroid analogue less readily than it does the parent hormone. Examination of peritoneal cavities of all rats at the conclusion of the 48-hour collection period revealed no evidence of incomplete absorption of either agent.

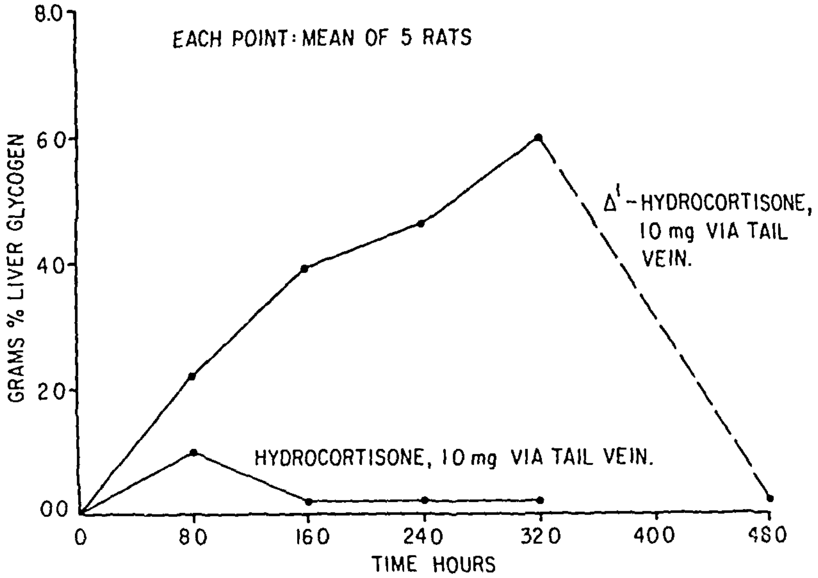

FIG. 2. Effect of intravenous administration of hydrocortisone and Δ^1-hydrocortisone (prednisolone) on liver glycogen deposition. Rats were adrenalectomized 3–4 days, fasted 24 hr. before injection, and the fasting continued to the end of the experiment.

DISCUSSION AND CONCLUSIONS

Results of both *in vitro* and *in vivo* studies in the rat indicate that metabolism of prednisolone is considerably less efficient than that of hydrocortisone. *In vitro* results reported here are in essential agreement with those reported by Schriefers for rat liver slices (3).

Direct addition of steroid substrates to liver slices and measurement of specific changes on the steroid molecule following one particular time period of observation is insufficient evidence for differences in metabolism of various compounds. Unless specific cofactor, coenzyme and cosubstrate requirements of a particular enzymatic reaction are thoroughly understood, differences, or lack of them, following addition of various materials mean very little with regard to the interpretation of significance of data. Certain subleties exist in experiments of this type, and acquaintanceship with various analytical procedures involved are requisite for a thorough analysis of the multitude of factors involved in such a complex relationship.

Obviously, *in vitro* observations alone are insufficient evidence for the conclusion that a series of similarly related events occur in the intact organism. However, when such data are coupled with many other types of analyses, using the intact animal, a valid conclusion may be warranted.

The question of whether a decreased rate of steroid metabolism is solely responsible for enhanced biological activity of prednisolone remains unanswered. However, a slower rate of inactivation or excretion of compound from the organism necessarily implies an enhanced biological efficacy, since more steroid becomes available to responsive tissues for a longer period.

Difficulties may be encountered when attempts are made to analyze differences in either steroid metabolism rates or steroid biological effects when one rigidly applied artificial time period and steroid concentration is imposed upon the system being examined. For example, 2-methyl-9α-fluorohydrocortisone is thirty-three times more potent as a glycogenic agent than is hydrocortisone; prednisolone is three times more active. However, when all three compounds are administered *subcutaneously* in equimolar amounts (1 mg.) and livers analyzed for liver glycogen deposition 8 hours later, amounts of glycogen are approximately the same in all cases (18). One would be tempted to conclude that the three compounds are exactly equivalent on a biological basis. However, when analyzed as a function of *time*, prednisolone and 2α-methyl-9α-fluorohydrocortisone produce glycogenic responses which persist for many hours after glycogenic response to hydrocortisone has disappeared (18). Furthermore, when administered in "biologically equivalent" amounts (hydrocortisone, 1.0 mg.; prednisolone, 0.3 mg.; and 2α-methyl-9α-fluorohydrocortisone, 0.03 mg.), the glycogenic time curves become superimposable for these three steroids (18). These latter results further emphasize the probability that not only do retarded rates of steroid metabolism partially explain increased activity of certain steroids, but that other unknown mechanisms may be operating to bring about increased biological efficacy.

Acknowledgment

The author gratefully acknowledges the confirmatory analytical data collected by Mr. Lester Reineke concerning amounts of hydrocortisone and prednisolone excreted in the urine of rats following administration of each steroid.

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A METHOD OF ASSAY OF ANTITUMOR ACTIVITY USING A RAT MAMMARY FIBROADENOMA

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ABSTRACT

An assay method for investigation of factors which influence growth rate of a benign mammary fibroadenoma of female rats has been described. Variations in number of absolute tumor "takes," final tumor size and variable growth rates, necessitates employment of a definitely established, growing tumor for routine assay purposes.

Marked differences exist between response of mammary fibroadenomas to early treatment and treatment after tumors are growing autonomously. Since established tumors more nearly simulate the tumors dealt with in clinical medicine, studies of tumors of this type may provide correlations between laboratory and clinical observations.

Ovariectomy retards growth rate of established mammary fibroadenomas; adrenalectomy induces only a slight inhibitory effect; but both procedures combined result in greater inhibition of tumor growth.

Estradiol cyclopentylpropionate, at dose levels which inhibit growth rate of *initially* transplanted tumors, stimulates growth rate of *established* tumors.

Progesterone, which stimulates tumor growth if administered from day 1 of transplantation, has no effect on subsequent tumor growth if administration is initiated after tumors are well established.

Hydrocortisone, if given in amounts adequate to prevent body growth, has no effect on growth of established tumors. However, administration of similar amounts of the same hormone for 5 days prior to tumor implantation results in greater final tumor size 65 days later.

Testosterone propionate, reference standard for this assay, has been found to consistently inhibit tumor growth but never induces regression. Testosterone cyclopentylpropionate has a biphasic effect on tumor growth, stimulating growth at low doses, inhibiting at higher doses. Tumor inhibitory effects of testosterone propionate are accompanied by ovarian and adrenal involution and preputial gland and uterine stimulation.

Four groups of controls are required for an assay of this type: initial tumor implant weights (1 of every 10 implants), pretreatment controls (35 days following implantation), final controls (65 days following implantation) and hormonal controls (1.0 mg. of testosterone propionate each day from day 35 to day 65).

HUGGINS *et al.* (1, 2) recently described experimental conditions for study of a mammary tumor of female rats. This tumor, a benign mammary fibroadenoma, responds in rather consistent fashion to certain

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hormonal alterations and glandular extirpations. Since effects of hormonal therapy and glandular removal on mammary tumor development have been learned almost exclusively from studies in man, use of an experimental tumor which responds in similar fashion becomes an important adjunct to this area of research.

Huggins *et al.* (2) have reported that most steroid hormones capable of inducing retardation of mammary fibroadenoma development also possess estrogenic or androgenic properties. Studies were initiated in this laboratory to determine if response of benign mammary fibroadenomas to steroid hormones was merely a reflection of other characteristic hormonal responses or whether this type of tumor represented an analytical tool which would enable the investigator to discover steroidal antitumor agents which do not induce a characteristic pattern of hormonal activity in the host. That this latter possibility exists has been suggested by the work of Huggins *et al.* (3, 4) who showed the inhibitory influence of 3-methylcholanthrene on both the experimental tumor and on spontaneous neoplasms in man. 3-Methylcholanthrene administration does not induce the characteristic hormonal pattern of activity observed following administration of androgens or estrogens.

Benign mammary fibroadenomas occur spontaneously in 50–65% of all female Upjohn Wistar (5) and Sprague-Dawley (1) rats after the age of 18 months. Both types of tumor can be transplanted into females of the same strain. Transplants grow slowly, if at all, during the initial three weeks following transplantation (latent period) then begin to grow more rapidly in a progressive manner. Tumors do not grow readily in male rats, in hypophysectomized or ovariectomized females, except under extraordinary circumstances. Ovariectomy retards growth rate of the rapidly growing tumor; adrenalectomy has a slight inhibitory effect; and removal of both hormonal sources induces greater inhibition of tumor growth than either procedure alone. Growth rate of benign mammary fibroadenomas is also retarded by administration of estrogens or androgens in appropriate amounts.

METHODS

Since the percentage of animals in which transplanted tumors grow varies from one series of experimental animal to another, and since the number of "takes" per rat varies when multiple transplants are employed, procedures described by Huggins *et al.* (1) have been slightly modified in the interest of greater assay reproducibility.

The original tumors were obtained from Dr. Huggins' laboratory in February, 1957; were transplanted to a large number of female Sprague-Dawley rats and have subsequently produced a tumor strain used successfully in our laboratory for more than twelve months.

Female Sprague-Dawley rats (45–50 days old, 140–160 gm.) are anesthetized with 1.0 cc. of 1% cyclopal by intraperitoneal injection. Incisions are made in the back of each animal, and channels in the subcutaneous connective tissue made with surgical forceps to about ½ inch caudal to each front leg on both sides. One operator removes "seed" tumors (one "seed" tumor-bearing animal sacrificed for every one hundred recipients) by cutting slices of uniform thickness and punching uniform pieces of tumor (70 ± 20 mg.) with nasal cutting forceps. Transplants (one on each side) are quickly inserted into previously made channels and incisions are closed with surgical clips.

The animals are housed in cages in constant temperature, constant humidity animal quarters. Animals are allowed tap water and Purina lab chow *ad libitum*. Two operators can carry out the entire operation described above on two to three hundred rats in 1½ to 2 hours.

Tumors are allowed to grow for 35 days; animals are then palpated for actively growing tumors. Those with extremely small or no tumors are discarded; those with large tumors are set aside for subsequent "seed" tumors. The remaining animals are randomized in "haphazard" fashion by placing in large carrying cages, removing animals, and dividing into dosage groups of eight to ten animals. Test compounds are administered daily, orally or subcutaneously, as finely dispersed suspensions in 0.5 or 0.2 ml. of carboxymethylcellulose vehicle. Treatment is continued for 30 days, after which animals are sacrificed and autopsied. The eight to ten animals used for each experimental point usually provide 16–18 tumors of rather uniform size. Three groups of controls are used in every experiment: an *initial control*, ten tumor-bearing animals sacrificed at the end of the 35-day growth period; a *final control*, consisting of untreated animals in which tumors have grown for 65 days; and a *hormone control*, consisting of ten animals to which 1.0 mg./day of testosterone propionate has been administered subcutaneously for 30 days. The last control is to insure that the tumors in the experimental rats are hormone-sensitive and have not mutated to the Huggins "Class II" (3) hormone-insensitive fibroadenoma.

RESULTS AND DISCUSSION

A characteristic growth rate curve of the tumor is illustrated in Figure 1. It is apparent that growth proceeds slowly during the first 21 days following transplantation. The 35-day period was chosen for experimental studies because tumors are easily inspected visually and manually at this time.

That the benign mammary fibroadenoma has a variable growth rate, when data from one experiment are compared with those from another, is well illustrated in Table 1. These results were compiled from separate assays during a two-month period. It is evident that adequate numbers of control animals must be examined both at the end of the 35-day and 65-day growth periods for each experimental series.

In Table 2 the number of tumor "takes" for several series of animals during a three-month period is shown. It is evident that considerable variation (45–75%) in number of tumor "takes" exists. It is equally evident that with such variation in number of tumor successes, treatment of tumor-bearing animals from the beginning of the transplantation period becomes a difficult problem. That is, difficulties might arise in the interpretation of significance of data if, in an experimental group, the number of tumor "takes" were 75% and those of the control group only 45%. For this reason we have elected to determine the influence of various agents on growth rate of mammary fibroadenomas only when absolute assurance is made that all treated and untreated animals have one or two actively growing tumors. Occasionally we have had a complete failure of tumor "takes" in an entire series of experimental animals. Explanations for these complete failures are

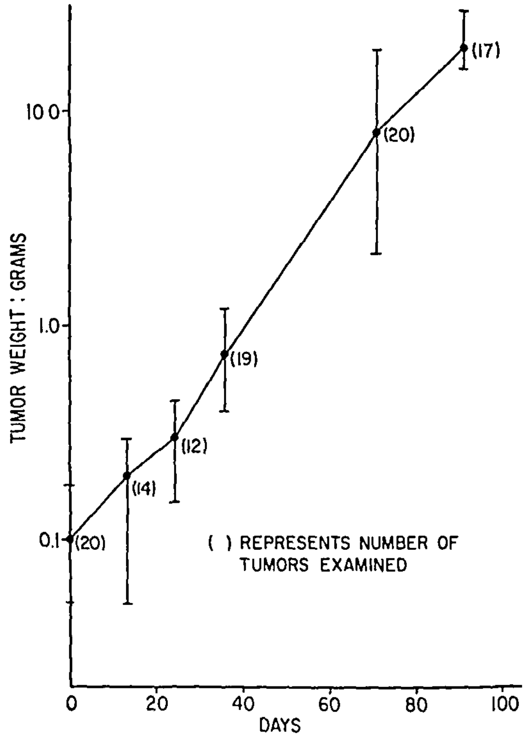

FIG. 1. Growth rate of mammary fibroadenoma.

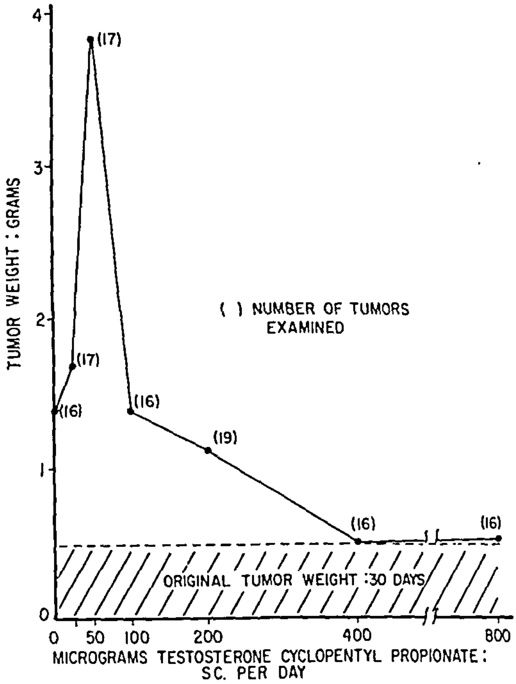

FIG. 2. Response of established mammary fibroadenoma to varying amounts of testosterone cyclopentylpropionate. Note: Final control tumor weights were lower than usual because these data were obtained in early days of investigations with tumor; at that time a rapidly growing "seed" tumor had not yet been discovered.

not available. However, their frequency does not obviate the use of this tumor as an analytical testing method.

Since the benign mammary fibroadenoma responds to androgenic agents, *testosterone propionate* was chosen as reference standard. In Table 3 data are shown to illustrate results obtained during a six-month period. It is obvious that testosterone propionate administration produces a consistent pattern of tumor inhibition (94–98%). Regression of the established mam-

TABLE 1. VARIATION IN SIZE OF MAMMARY FIBROADENOMAS 35 AND 65 DAYS FOLLOWING TRANSPLANTATION

Experiment No.	35-day tumors			65-day tumors		
	No. rats	No. tumors	Mean tumor weight (gm.)	No. rats	No. tumors	Mean tumor weight (gm.)
1040	10	19	.697	10	16	8.400
1037	10	19	.605	10	17	11.400
1035	10	16	.879	10	14	11.203
1034	10	17	.977	10	18	10.210
1032	10	17	.459	10	19	4.430
1028	10	19	.436	10	16	7.960

TABLE 2. VARIATION IN NUMBER OF TUMOR "TAKES" FOLLOWING TRANSPLANTATION OF BENIGN MAMMARY FIBROADENOMA

Experiment No.	No. animals	No. tumors transplanted	No. animals with tumors after 35 days	Per cent
1040	200	400	130	65
1037	200	400	90	45
1035	200	400	120	60
1034	200	400	102	51
1032	200	400	128	64
1029	200	400	135	67
1028	200	400	150	75
1026	200	400	95	47
1024	200	400	150	75
1020	156	312	107	68
1017	202	404	134	66
1015	200	400	155	77
1018	200	400	130	65
Total	2558	4116	1626	Avg. 64

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON GROWTH RATE OF MAMMARY FIBROADENOMA*

Treatment	No. animals with tumors (35 days)	No. takes 35 days	No. takes 65 days	Mean tumor weight (gm.)	Per cent inhibition
Initial Control	10	18		0.602	
Final Control	10		16	8.950	
Testosterone propionate†	10		17	1.030	94.9
Initial Control	10	19		0.356	
Final Control	10		17	4.420	
Testosterone propionate	10		20	0.568	94.8
Initial Control	10	19		0.913	
Final Control	10		17	6.070	
Testosterone propionate	10		18	1.040	97.9
Initial Control	10	16		0.950	
Final Control	10		15	10.210	
Testosterone propionate	10		14	1.130	98.9
Initial Control	10	15		1.062	
Final Control	10		14	17.880	
Testosterone propionate	10		11	1.250	98.9
Initial Control	10	17		0.977	
Final Control	10		13	10.200	
Testosterone propionate	10		20	1.180	97.9

* Selected at random from series of controls for period of 6 months.
† 1.0 mg./day for 30 days, subcutaneously.

mary fibroadenoma has never been observed following administration of testosterone propionate. Microscopic examination of tumors from testosterone propionate-treated animals reveals no significant alteration in morphological characteristics.[1] During a twelve-month period five animals from 202 testosterone-treated female rats were observed to possess resistant tumors.

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[1] Morphological examination of all tumors was made by Dr. John Clark, The Upjohn Company. Results will be published by Dr. Clark and the senior author in another communication.

A biphasic relationship exists between tumor development and dose of testosterone cyclopentylpropionate. That is, low doses of this particular steroid (25–50 μg./day) *stimulate;* higher doses *inhibit* tumor growth (Fig. 2).

Table 4 shows the effect of varying amounts of estradiol on growth rate of tumors and various end organs of female rats. It is evident that 0.2 to 48.0 μg./kg. of this material stimulates tumor development. The effect of estrogen administration under these conditions is different from that reported following estradiol cyclopentylpropionate administration beginning from day 1 of tumor transplantation (Fig. 3). These results may indicate differences in sensitivity of the established versus the newly-transplanted tumor. Larger doses of estradiol may be required to inhibit the established, rapidly growing tumor.

These latter results prompted a re-examination of possible differences between treatment of animals beginning at the time of tumor implantation and treatment initiated after tumors were established, i.e., growing autonomously. In Figure 3 results of treatment of tumor-bearing animals with various steroids are illustrated. All animals were treated from day 1 to day 90 following transplantation. It is important to note that 17-methyltestosterone, 9α-fluoro-11β-hydroxy-17-methyltestosterone (*Halotestin*) and estradiol cyclopentylpropionate (2 μg./day) all prevented this tumor from growing. The effect of estrogen under these conditions differs from that obtained when estradiol cyclopentylpropionate in equivalent amounts was administered following establishment of the benign mammary fibroadenoma (Fig. 4).

It will also be noted that amounts of hydrocortisone (2 mg./day, every

TABLE 4. EFFECT OF ESTRADIOL ON MAMMARY FIBROADENOMA DEVELOPMENT AND VARIOUS ENDOCRINE ORGANS OF THE FEMALE RAT

	No. tumors	BW gain (gm.)	Uterus (mg.)	Ovaries (mg.)	Preputial gland (mg.)	Adrenal (mg.)	Tumor wt. (gm.)	Per cent inhibition
Initial Control	19						0.559	
Final Controls	17	+16	443	109	77	58	7.460	
Estradiol								
0.05 μg./kg.	12	+24	467	110	108	68	7.980	+ 8
0.10 μg./kg.	12	+20	474	102	88	68	9.900	+36
0.20 μg./kg.	12	+19	478	118	69	64	15.570	+120
0.50 μg./kg.	12	+22	529	100	120	69	15.780	+121
1.50 μg./kg.	12	+26	440	93	86	62	15.040	+110
3.00 μg./kg.	15	+15	418	90	77	67	13.870	+93
6.00 μg./kg.	11	+ 7	441	86	83	64	14.120	+97
12.00 μg./kg.	14	+ 9	446	87	88	71	7.830	+ 5
24.00 μg./kg.	15	+13	623	73	99	74	10.020	+37
48.00 μg./kg.	13	0	759	63	81	67	9.090	+24
80.00 μg./kg.	14	− 4	688	56	97	68	6.340	−16

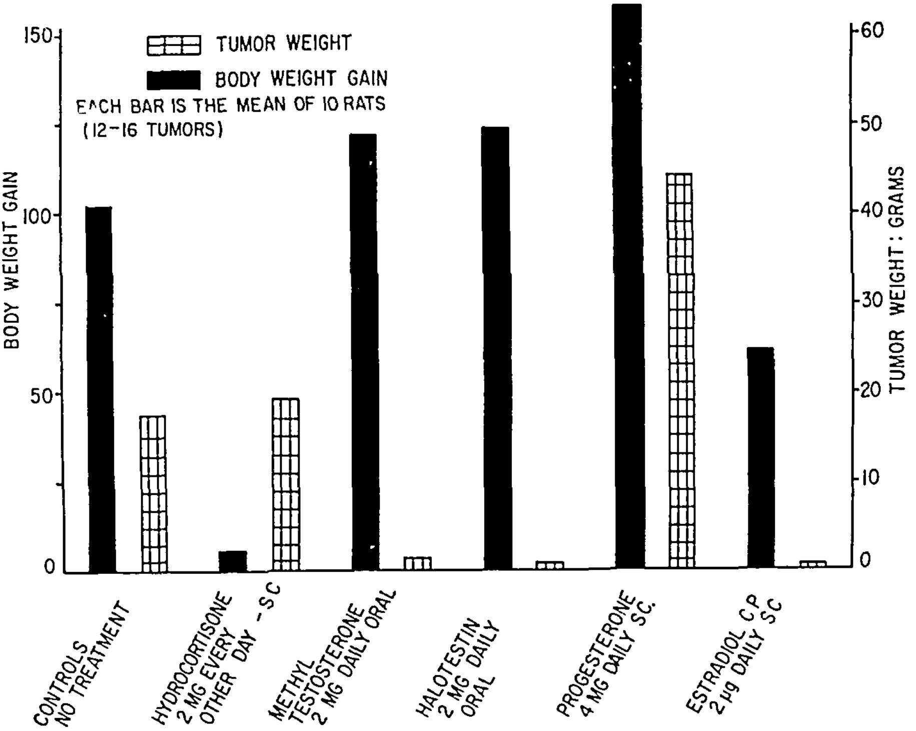

FIG. 3. Effect of various steroids on growth rate of the established mammary fibroadenoma.

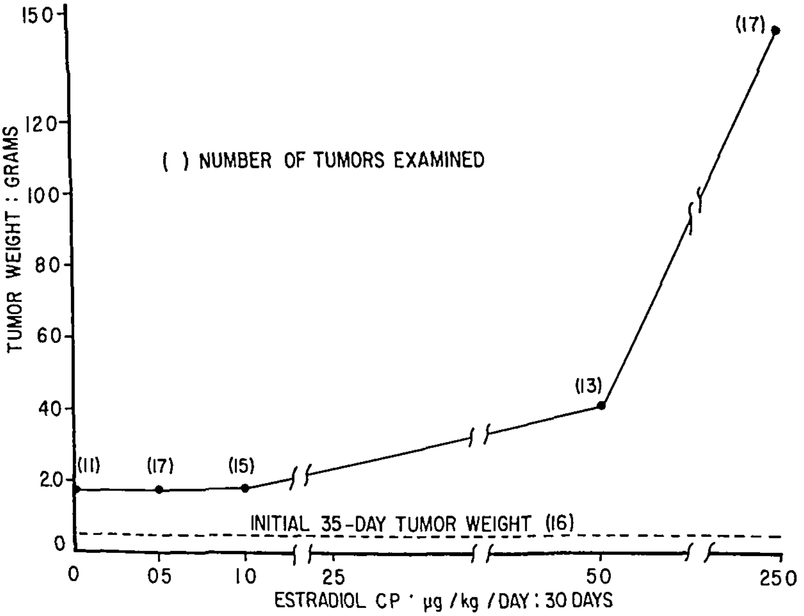

FIG. 4. Effect of estradiol cyclopentylpropionate on the growth rate of the established mammary fibroadenoma. Note: Final control tumor weights were lower than previous series because same slow-growing "seed" tumor from data in Fig. 2 used for initial implantation.

TABLE 5. EFFECT OF PRIOR ADMINISTRATION OF HYDROCORTISONE ON SUBSEQUENT TUMOR GROWTH

No. animals	No. tumors	Treatment	Tumor size	
			35 days	65 days
10	16	None	0.972	
10	18	None		20.200
20	38	2.0 mg. hydrocortisone per day, 5 days before implantation		51.530
10	17	2.0 mg. hydrocortisone per day, 5 days before implantation	0.875	

other day), sufficient to completely prevent body weight gain, had little influence on tumor growth. These results are in direct contrast to those obtained following administration of hydrocortisone *prior* to tumor transplantation. Hydrocortisone (2 mg./day) was administered for 5 days before tumor transplantation, then stopped; tumors were implanted on day 5 and examined 65 days later. Under these circumstances, tumors from hydrocortisone-treated animals were two to three times larger than control tumors from untreated animals (Table 5). These results are important from the standpoint of possible relationships which may exist between host resistance and subsequent mammary fibroadenoma development.

Results obtained with progesterone are in agreement with those reported

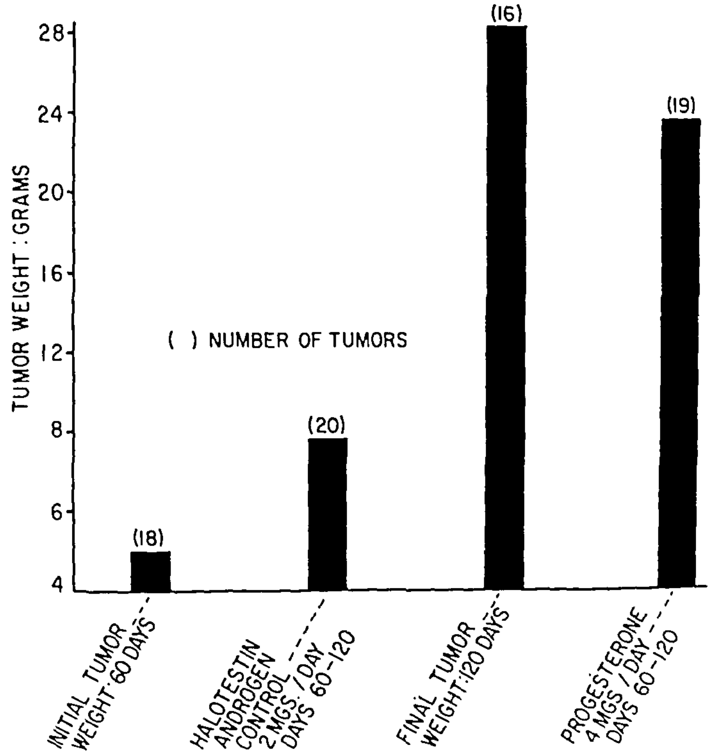

FIG. 5. Effect of progesterone administration on established mammary fibroadenoma.

by Huggins (1) who showed that treatment of animals with progesterone beginning at the time of tumor transplant resulted in considerable enhancement of tumor growth rate (Fig. 3). However, when equivalent amounts of progesterone were administered to animals bearing rapidly growing tumors (60 days following transplantation) for a period of 60 days (day 60–day 120), no effect was observed (Fig. 5). These results further emphasize differences which exist between treatment of animals with freshly-transplanted tumors and animals with well-established tumors. Either tumor growth is sufficiently autonomous or rapid during its later proliferative

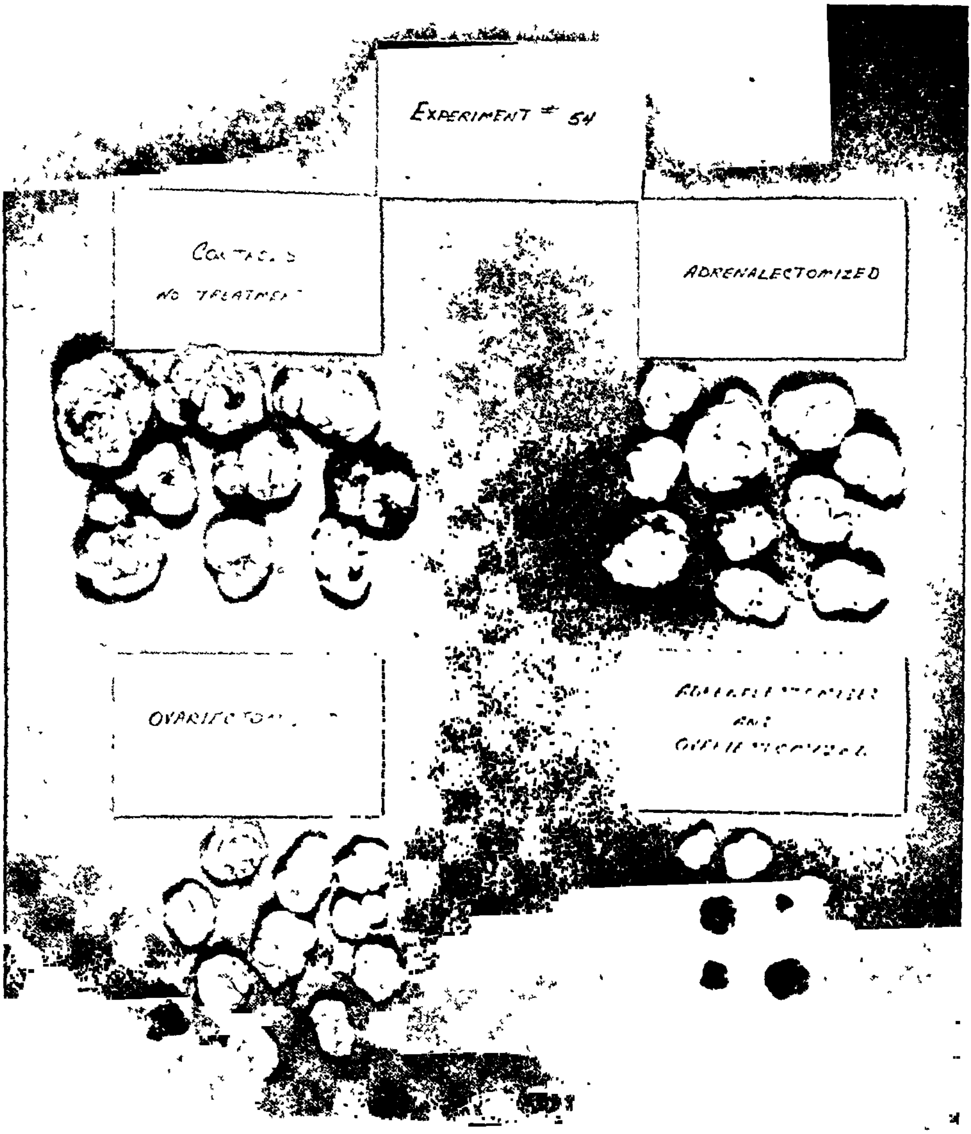

FIG. 6. Influence of adrenalectomy, ovariectomy and adrenalectomy plus ovariectomy on the growth of the established mammary fibroadenoma.

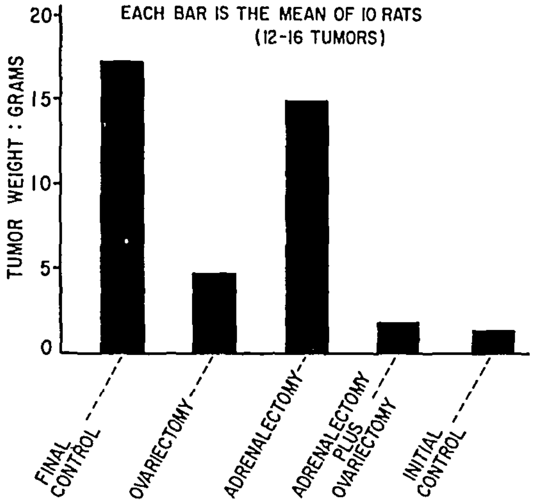

FIG. 7. Influence of adrenalectomy, ovariectomy and adrenalectomy plus ovariectomy on the growth of the established mammary fibroadenoma.

phases to prevent further influence by progesterone, or the pro-tumor effect of this agent is limited entirely to the host. This unknown effect may permit better establishment of tumor during its latent or early proliferative phases of development. However, prior administration of progesterone to experimental animals before tumor implantation (4 mg. for 7 days) did not result in greater number of tumor transplant "successes."

In Figures 6 and 7 results are presented to show the effect of removal of ovaries and/or adrenals on tumor growth. Adrenalectomized animals were maintained on 0.9% NaCl and 0.2 mg. hydrocortisone per day. It is evident that combined adrenalectomy-ovariectomy results in greater tumor inhibition. Neither procedure induces regression.

Testosterone propionate administration induces an increase in preputial gland growth while exerting an inhibitory influence on mammary tumor development (Table 6). The effect of varying amounts of testosterone propionate on ovaries, adrenals and uterus are also shown in Table 6. Simultaneous inhibition of both ovarian and adrenal secretory activity may explain, in part, the antitumor properties of this compound.

Since increased growth rate of preputial glands following administra-

TABLE 6. EFFECT OF TESTOSTERONE PROPIONATE ON TUMOR AND HORMONAL TARGETS OF FEMALE RATS

Testosterone propionate* (mg./kg./day, 30 days)	Tumor wt. (gm.)	Uterus wt. (mg.)	Preputial glands (mg.)	Adrenal (mg.)	Ovary (mg.)
0	8.210	582	99	62	90
0.5	5.420	280	152	62	49
1.0	4.890	339	161	51	38
2.5	0.850	820	229	48	44
5.0	0.620	901	321	48	56

* 10 rats per dose, 12–13 tumors per group of rats. Pretreatment tumor weight .621 gm. Testosterone propionate administered subcutaneously between days 35 and 65 after tumor transplantation.

TABLE 7. EFFECT OF TESTOSTERONE PROPIONATE ON GROWTH OF SEMINAL VESICLES AND LEVATOR ANI MUSCLES OF CASTRATE MALE RATS

Testosterone propionate (mg./kg./day, 20 days)	No. rats	Seminal vesicle (mg.)	Levator ani (mg.)
0.25	10	74	79
0.50	10	141	116
1.00	10	212	159
2.00	10	280	171
4.00	10	331	189
8.00	10	349	179
None	10	10	49

Note: These data were obtained following subcutaneous administration of testosterone propionate for 20 days to 27 day old castrate male rats (7).

tion of androgen is presumed to be an index of its "masculinizing" activity (6), the response of these organs appears to be the most critical or sensitive index of androgenicity. However, this statement is merely a working hypothesis and does not imply that for a compound to stimulate preputial gland development it must also be an androgen. Growth hormone and ACTH have also been shown to stimulate growth of preputial glands (8, 9). For comparative purposes, the androgenic and "myotrophic" activities of testosterone proprionate are illustrated in Table 7. Comparison of Table 6 with Table 7 reveals that amounts of testosterone propionate necessary to significantly inhibit mammary fibroadenoma growth rate are markedly androgenic and also significantly stimulate growth rate of preputial glands.

Acknowledgment

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INHIBITION OF MAMMARY FIBROADENOMA OF FEMALE RATS BY STEROIDS OF THE ANDROSTANE SERIES

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ABSTRACT

Results have been presented to suggest that steroids can inhibit benign mammary fibroadenoma development without exerting significant androgenic or estrogenic effects in experimental animals. Preliminary observations suggest that the antitumor efficacy of these agents may depend upon their ability to inhibit certain secretory functions of the adenohypophysis. However, direct effects of steroids on tumor growth rate in the absence of the pituitary have not been determined. Final resolution of these questions awaits more detailed investigation in experimental animals and man.

Compared with 17-methyltestosterone, 11α-hydroxy-17-methyltestosterone and 9,11-epoxy-17-methyltestosterone have decreased androgenic activity while their antitumor activity is in a large measure the same. Compared with 19-nortestosterone, 2-methyl-19-nortestosterone has less so-called "masculinizing" ability in female rats, but antitumor activity is substantially the same.

Possible mechanisms regarding ability of these steroids to inhibit growth rate of mammary fibroadenomas in female rats cannot be stated with certainty at this time.

GROWTH rate of the Huggins tumor (a benign mammary fibroadenoma of rats) is retarded by a number of androgenic steroids (1, 2). This tumor will not grow in male rats, will not grow in hypohysectomized rats (except rarely), and growth rate is decreased by simultaneous removal of both adrenals and ovaries (3). The benign fibroadenoma has never been observed to undergo regression, nor are morphological characteristics significantly altered following administration of potent androgens (3). Growth rate is merely retarded by these agents. Growth rate is accelerated by administration of appropriate amounts of progestational and estrogenic steroids (2). However, these latter compounds do not markedly accelerate tumor growth when administered to hypophysectomized rats (2). If transplanted mammary fibroadenomas are allowed to grow for a number of weeks, followed by ovariectomy and/or hypophysectomy, they will stop growing but tumor regression is not induced by these procedures.

Huggins (2) has reported that most C-19 steroids which retard growth rate of the benign fibroadenoma also possess varying degrees of estrogenic

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or androgenic activity. Since hypophysectomy and ovariectomy retard tumor growth, an agent which effectively blocks ovarian or pituitary function, which also possesses minimal androgenic or estrogenic activity, would appear to be a desirable adjunct to treatment of patients with mammary carcinoma.

METHODS

A benign mammary fibroadenoma assay method has been described previously (3). Techniques used here are modifications of those recently reported by Dr. Huggins (1). Essentially, this procedure involves the use of an established tumor which has grown for 35 days prior to steroid administration. Two tumors were usually present in each experimental animal; 8–10 animals were employed for each experimental point. Control tumors were weighed 35 days and 65 days following implantation. These were designated initial controls and final controls respectively. An additional group, to which 1 mg. of testosterone propionate per day had been administered (day 35–day 65), was employed in every experimental series and was designated hormonal controls. Steroids were administered daily (day 35–day 65) either orally or subcutaneously as finely dispersed suspensions in 0.5 cc. or 0.2 cc. of carboxymethylcellulose.

Androgenic and "myotrophic" activities of steroids were determined by analysis of seminal vesicle and levator ani muscle weight changes in castrate male rats (4). Estrogenic activities of various steroids were determined by uterine weight changes in gonadectomized female rats.

RESULTS

Results obtained in the hormonal control group are indicated in Table 1. During the period in which the following experiments were undertaken (December, 1957–April, 1958) the benign mammary fibroadenoma was consistently responsive to testosterone propionate.

Testosterone propionate, at dose levels necessary to significantly retard tumor growth, also stimulates growth rate of preputial glands and uteri (Table 2). Growth of preputial glands of female rats following administration of androgens has been selected as an index of "masculinizing" properties of various compounds (5). That this assumption may not be strictly correct is indicated by studies which show that these end organs are also stimulated by growth hormone and ACTH (6, 7, 8, 9).

Investigations of antitumor activity of C-19 steroids revealed several interesting agents. Among these interesting compounds were 9,11-epoxy-17-methyltestosterone (U-5518) and 11α-hydroxy-17-methyltestosterone (U-5662). When compared with testosterone propionate, these two compounds inhibited mammary fibroadenoma development, but had no effect on uterine development (Fig. 1). 9,11-Epoxy-17-methyltestosterone did not stimulate growth rate of preputial glands at any dose level, while 11α-hydroxy-17-methyltestosterone did. Although the latter agent appeared to be a more potent antitumor agent than testosterone propionate, it did not stimulate growth of preputial glands as markedly as testosterone propionate (Fig. 1). Oral activity of these two compounds is illustrated in Figure 2. When compared to 17-methyltestosterone, U-5518 was equally

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ADMINISTRATION (5 MG./KG.) ON GROWTH RATE OF ESTABLISHED BENIGN MAMMARY FIBROADENOMA

No. animals	No. tumors	Control tumors 35 Days (gm.)	Control tumors 65 Days (gm.)	Testosterone propionate (1 mg./day, 30 days)	Per cent tumor Inhibition
10	19	.478			
10	15		6.029		
8	15			.680	96.4
10	19	.592			
8	15		19.426		
8	13			.876	98.4
10	19	.557			
10	15		11.570		
8	16			.901	96.9
10	18	.816			
10	20		12.830		
10	20			.954	98.9
10	16	.980			
10	12		10.945		
10	18			.701	100.0
10	19	.557			
10	12		6.934		
10	15			.944	97.7
10	20	.473			
10	14		8.316		
10	14			1.794	83.2
10	15	.383			
10	15		9.160		
10	14			1.330	89.2
5	10	.458			
8	11		11.059		
8	12			1.203	92.9
10	19	.755			
10	20		3.082		
10	20			.761	100.0
10	19	.373			
10	14		2.744		
10	19			.568	91.8

TABLE 2. EFFECT OF VARYING AMOUNTS OF TESTOSTERONE PROPIONATE ON GROWTH RATE OF BENIGN MAMMARY FIBROADENOMA AND VARIOUS ORGANS OF INTACT FEMALE RATS

Dose mg./kg./day SC.	No. Animals	No. Tumors	Body weight gain (gm.)	Uterus (mg.)	Ovaries (mg.)	Preputial gland (mg.)	Adrenals (mg.)	Tumor wt. (gm.)	% Inhibition
Initial Controls	10	19						0.697	
Final Controls	8	13	+36	571	91	87	60	8.400	
0.5	8	16	+50	271	50	151	62	5.700	35
1.0	8	16	+54	384	36	158	53	5.360	40
2.5	8	12	+56	842	44	230	50	0.834	99
5.0	8	13	+55	900	52	307	48	0.720	100

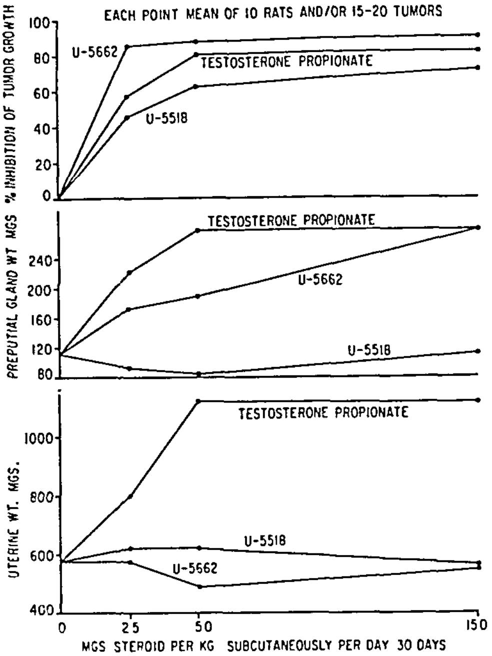

FIG. 1. Effect of subcutaneously administered *testosterone propionate*, U-5518 and U-5662 on growth rate of established mammary fibroadenoma, preputial glands and uteri of intact female rats.

as effective as an antitumor agent, but did not stimulate significantly growth rate of preputial glands.

Androgenic and "myotrophic" effects of these two steroids were compared in castrate male rats. Analysis of data shown in Figure 3 reveals that

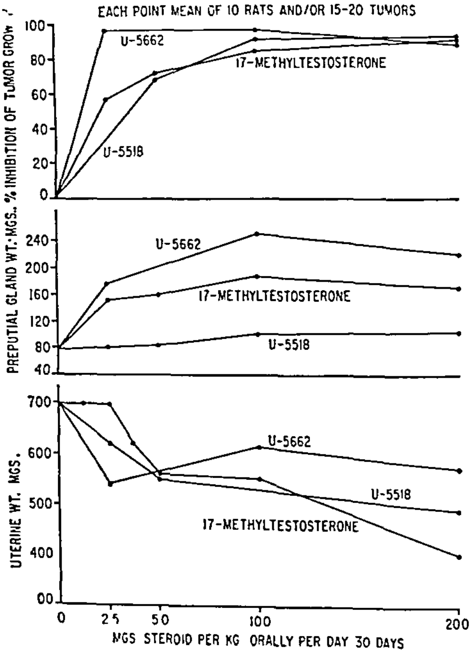

FIG. 2. Effect of orally administered 17-methyltestosterone, U-5662 and U-5518 on growth of established mammary fibroadenoma, preputial glands and uteri of intact female rats.

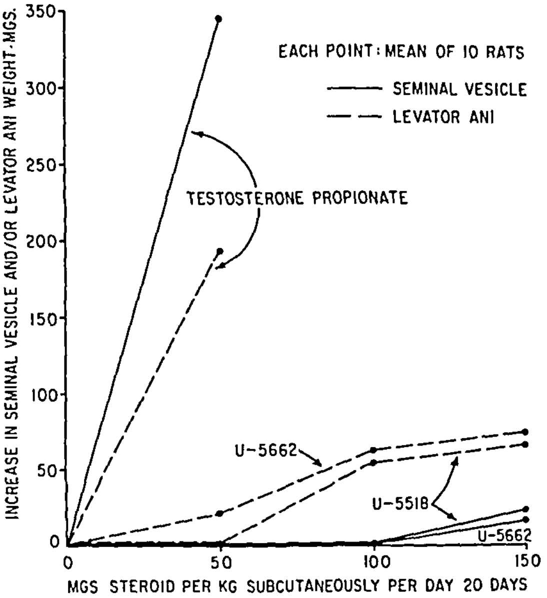

FIG. 3. "Myotrophic" and androgenic potency of testosterone propionate, U-5662 and U-5518.

both these steroids are less than 5% as active as testosterone propionate as androgenic or "myotrophic" agents. In addition, dose levels which produce significant anti-tumor activity in female rats were not androgenic in male rats (Figs. 1 and 3).

When administered at the higher dose levels (15 mg./kg.) to gonadectomized female rats, neither 9,11-epoxy-17-methyltestosterone nor 11α-hydroxy-17-methyltestosterone exhibited a significant degree of uterotrophic activity. However, the 11α-hydroxy compound augmented slightly the growth stimulatory effects of estradiol on the uterus (Table 3).

Since both 11α-hydroxy-17-methyltestosterone and 9,11-epoxy-17-methyltestosterone inhibited tumor growth without appearing to possess a significant degree of either androgenic or estrogenic activity, it was of interest to determine their effects on other end organs. Results of these experiments are illustrated in Table 4. It is apparent that both compounds significantly retarded growth of ovaries and adrenals of tumor-bearing female rats.

It has been demonstrated previously that 15 mg./kg. of either of these latter compounds is less than 5% as androgenic as testosterone propionate

TABLE 3. UTEROTROPHIC AND ANTI-UTEROTROPHIC ACTIVITY OF 11α-HYDROXY-17-METHYLTESTOSTERONE (U-5662) AND 9,11-EPOXY-17-METHYLTESTOSTERONE (U-5518)

Preparation	No. rats	Dose per day (20 days)	Uterus (mg.)
A CMC Vehicle	10	0.2 cc.	22
B Estradiol	10	0.8 μg./kg.	143
C U-5518	10	15.0 mg./kg.	23
D U-5662	10	15.0 mg./kg.	19
B+C	10		143
B+D	10		164

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE, 9,11-EPOXY-17-METHYLTESTOSTERONE AND 11α-HYDROXY-17-METHYLTESTOSTERONE ON GROWTH OF VARIOUS ORGANS OF FEMALE RATS

No. rats	Compound	Dose (mg./kg.) 30 days	Uterine wt. (mg.)	Ovarian wt. (mg.)	Preputial glands (mg.)	Adrenals (mg.)	Mean tumor wt. (gm.)
10	TP	5.0	779	95	306	60	.980
10	U-5662	15.0	389	82	208	51	.670
10	U-5518	15.0	440	78	102	48	.510
10	CMC Vehicle	—	520	116	92	72	9.700

Note: Initial tumor weight: .429 gm. (10 animals, 18 tumors).

at 5 mg./kg. (Fig. 3). However, when similar amounts were administered in these experiments, both of these compounds appeared to be slightly more active than testosterone propionate with regard to their ability to inhibit ovarian and adrenal function.

During the studies detailed above, several preliminary observations suggested that marked differences existed with regard to the effects of other steroids on growth rate of benign mammary fibroadenomas. At 15 mg./kg. 19-nortestosterone inhibited mammary fibroadenoma development, but also induced marked increases in preputial gland weight. However, at equivalent dose levels, the 2-methyl compound (2-methyl-19-nortestosterone) was as effective as an antitumor agent, but failed to stimulate significantly growth rate or preputial glands (Table 5). Further analysis of these observations is illustrated in Figure 4. It is apparent that 2-methyl-19-nortestosterone is a potent antitumor agent with minimal activity with regard to its ability to stimulate preputial gland development. Its antitumor effectiveness may reside in its ability to inhibit pituitary gonadotropin secretion as reflected by decreased ovarian and uterine weight changes following its administration.

TABLE 5. EFFECT OF 19-NORTESTOSTERONE, 2-METHYL-19-NORTESTOSTERONE AND TESTOSTERONE PROPIONATE ON GROWTH OF BENIGN MAMMARY *FIBROADENOMA OF FEMALE RATS*

Preparation	Dose (mg./kg.)	No. animals	No. tumors	Preputial glands (mg.)	Tumor wt. (gm.)	Per cent inhibition
Final Controls	—	8	11	99	8.375	—
19-Nortestosterone	15	8	15	317	.980	100
2-Methyl-19-nortestosterone	15	8	11	103	.820	100+
Testosterone propionate	5	8	11	279	1.130	98
Initial Controls	—	10	16	—	.950	—

When 9,11-epoxy-17-methyltestosterone, 11α-hydroxy-17-methyltestosterone and 2-methyl-19-nortestosterone were administered at the 15 mg. /kg./day level to weanling male rats, results indicated that 2-methyl-19-nortestosterone was an effective gonadotropin suppressor. 9,11-Epoxy-17-methyltestosterone and 11α-hydroxy-17-methyltestosterone did not demonstrate this degree of gonadotropin suppression (Table 5a).

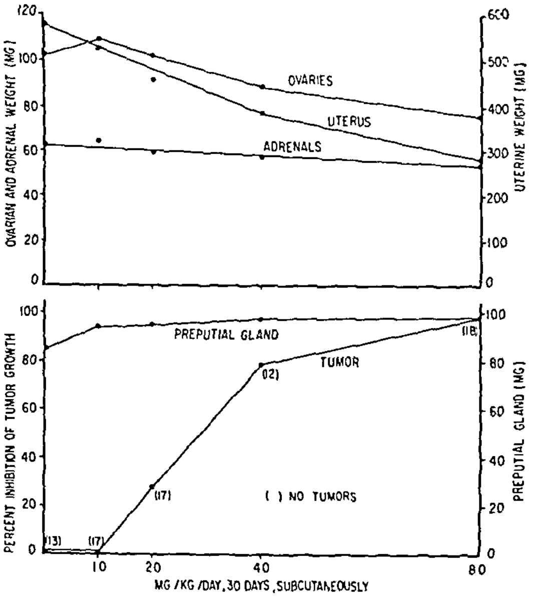

FIG. 4. Effect of 2-methyl-19-nortestosterone on growth rate of mammary fibroadenoma, preputial glands, uteri, ovaries and adrenals of intact female rats.

In order to further investigate the failure of 11α-hydroxy-17-methyltestosterone to inhibit gonadotropin secretion, it was administered in varying amounts to male weanlings for a 40-day period. When compared with testosterone propionate, it will be noted that 11α-hydroxy-17-methyltestosterone failed to inhibit testicular development (Table 5b). On the contrary, this steroid inhibited seminal vesicle growth rate, while testosterone propionate produced a marked increase in growth rate of this organ. These results are interesting with regard to antitumor effects of 11α-hydroxy-17-methyltestosterone which are significantly greater than those produced by testosterone propionate (Fig. 1). These latter results further emphasize the unique character of 11α-hydroxy-17-methyltestosterone.

Administration of 2-methyl-19-nortestosterone to female weanling rats resulted in marked depression of ovarian and uterine weights (Table 5c). These latter results also suggest that 2-methyl-19-nortestosterone is an effective gonadotropin suppressor at the particular dose levels employed.

Administration of 2-methyl-19-nortestosterone to castrate male rats

TABLE 5a. EFFECT OF 2-METHYL-19-NORTESTOSTERONE, 9,11-EPOXY-17-METHYLTESTOSTERONE, AND 11α-HYDROXY-17-METHYLTESTOSTERONE ON GROWTH RATE OF TESTES*

No. animals	Compound	Dose (mg./kg.)	Route	Testes (mg.)	Seminal vesicles (mg.)	Adrenals (mg.)	Body weight gain (gm.)
10	Controls	—	—	2274	269	33	113
10	9,11-Epoxy-17-methyltestosterone	15	SQ	2192	200	33	100
10	11α-Hydroxy-17-methyltestosterone	15	SQ	2187	201	30	117
10	2-Methyl-19-nortestosterone	15	SQ	1130	132	29	106

* Weanling male rats, 70–80 gm.; treated 40 days.

Table 5b. Effect of testosterone propionate and 11α-hydroxy-17-methyltestosterone on growth rate of seminal vesicles and testes of immature rats*

No. animals	Compound	Amount	Route	BW gain (gm.)	Seminal vesicles (mg.)	Testes (mg.)
5	TP	50 μg./day	SC	220	641	2820
5	TP	100 μg./day	SC	237	663	2460
5	TP	400 μg./day	SC	218	1040	1170
5	TP	1000 μg./day	SC	188	1516	2250
5	11-OH-MT	50 μg./day	SC	236	489	2800
5	11-OH-MT	100 μg./day	SC	232	509	3010
5	11-OH-MT	400 μg./day	SC	218	344	2800
5	11-OH-MT	1000 μg./day	SC	237	418	2900
5	Vehicle	2 cc./day		226	629	3100

* Compounds administered for 40 days. Weanling male rats, 25 days old at beginning of experiment.

Table 5c. Effect of 2-methyl-19-nortestosterone on growth rate of ovaries, adrenals and uteri of weanling female rats*

No. rats	Compound	Dose	Route	Preputial (mg.)	Uterus (mg.)	Ovaries (mg.)	Adrenals (mg.)	Initial BW (gm.)	Final BW (gm.)
10	None	—	—	61	271	73	49	55	174
10	2-Methyl-19-nortestosterone	15 mg./kg.	SQ	57	139	27	34	53	214

* Compound administered in 0.2 cc. carboxymethylcellulose per day for 40 days.

(15 mg./kg.) indicated that this compound was less than 1% as effective as testosterone propionate as an androgen and less than 2% as effective as a "myotrophic" agent (Table 6). As a uterotrophic agent this compound was 0.001% as active as estradiol (Table 7). This degree of uterotrophic

Table 6. Effect of 2-methyl-19-nortestosterone and testosterone propionate on growth rate of seminal vesicles and levator ani of castrate male rats

Compound	No. rats	Dose—SC (mg./kg.)	Seminal vesicle (mg.)	Levator ani (mg.)
Vehicle	10	0.1 cc.	6	41
2-Methyl-19-nortestosterone	10	15.0	34	97
Testosterone propionate	10	0.25	74	80
	10	0.50	140	118
	10	1.00	210	150
	10	2.00	277	170
	10	4.00	320	185
	10	8.00	351	161

Table 7. Effect of 2-methyl-19-nortestosterone and estradiol on uterine growth of gonadectomized female rats

Preparation	No. rats	Dose (20 days, SC)	Uterus (mg.)
A Vehicle	10	—	22
B Estradiol	10	0.4 μg./kg.	106
C 2-Methyl-19-nortestosterone	10	15.0 mg./kg.	57
B+C	10		118

activity does not appear to explain entirely the effectiveness of 2-methyl-19-nortestosterone as an antitumor agent, since somewhat larger amounts of estradiol are required to produce *stimulation* of established tumor growth (Table 4, previous paper). Amounts necessary to produce tumor inhibition have not been ascertained for the established tumor, but, since 15 mg./kg. of 2-methyl-19-nortestosterone is less active than 0.15 μg./kg. of estradiol, its minimal activity as an estrogen does not appear to explain its antitumor effects.

That a steroid can have minimal "masculinizing" properties while exerting maximal effects on growth rate of mammary fibroadenoma of experimental animals is well demonstrated in Figure 5. These data resulted from administration of 9α-fluoro-11β-hydroxy-17-methyltestosterone (*Halotestin*)[1] subcutaneously to animals bearing the Huggins tumor. When compared to Figures 1 and 2 it is apparent that 9α-fluoro-11β-hydroxy-17-methyltestosterone exerts maximal anti-tumor effects at dose levels which induce less stimulation of preputial gland growth than 17-methyltestosterone or testosterone propionate. However, in contrast to 17-methyltestosterone, 9α-fluoro-11β-hydroxy-17-methyltestosterone causes a marked increase in growth rate of uteri of intact female rats.

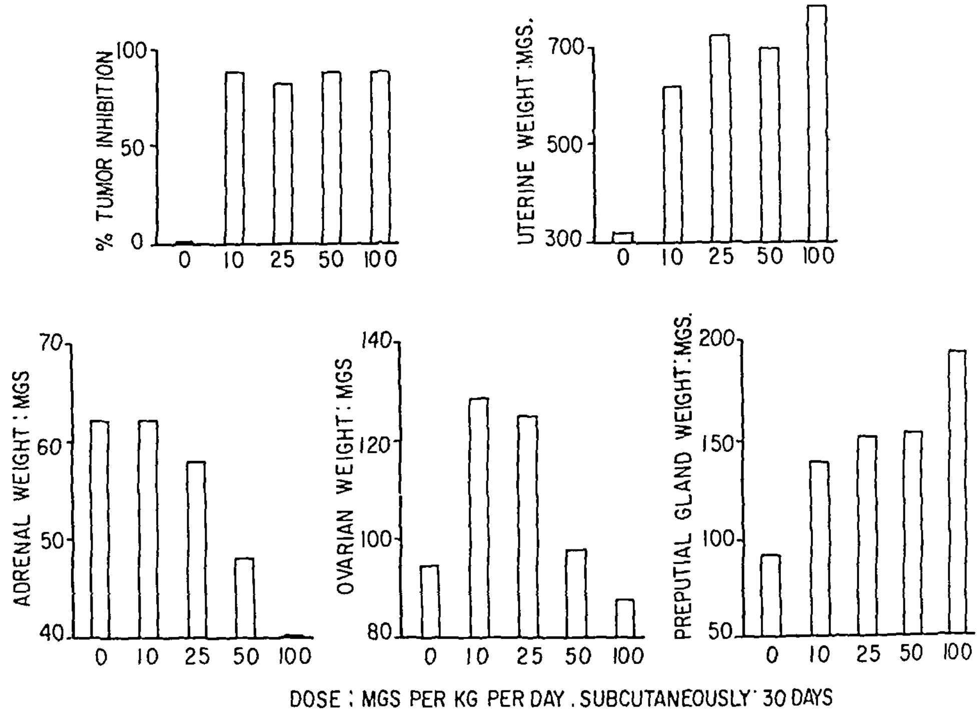

FIG. 5. Effect of 9α-fluoro-11β-hydroxy-17-methyltestosterone on uterus, ovaries, preputial glands, adrenals and benign fibroadenoma of intact female rats.

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[1] Registered trade mark, The Upjohn Company.

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THE MECHANISM OF INSULIN RESISTANCE IN THE KL STRAIN OF MICE: INACTIVATION OF INSULIN

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ABSTRACT

Insulin resistant (KL) mice, which tolerate 300–500 units of insulin without going into convulsions, inactivate insulin *in vivo* and *in vitro* at the same rate as control (BUB) mice which convulse after 50 units of insulin. The fasting blood sugar, glucose tolerance and pancreatic insulin content of the KL mouse is normal. After injection of small doses of insulin, the blood sugar response of the fed KL mouse is similar to that of the control BUB mouse. Following large doses of insulin, the blood sugar of control mice reaches the convulsive level within 2 hours. In the KL mouse, on the other hand, the blood sugar level declines but remains slightly above the convulsive level for 20–28 hours. These results are taken as evidence that insulin resistance in the KL mouse is not due to an increased insulinase activity, but to the ability of the animal to maintain a blood sugar slightly above the convulsive level for a long period after insulin administration.

MICE of the KL strain tolerate from 300–500 units of insulin without going into convulsions (1). Control BUB mice are more sensitive and convulse after doses below 50 units. The resistance to 300 units in the KL mouse is fully developed at 35 days of age, whereas older mice tolerate 500 units (1). Genetic studies suggest that the resistance may be due to about three pairs of additive recessive genes (2).

Previous investigations on the nature of insulin resistance in the KL mouse have shown that increased insulin excretion (3), increased glucagon secretion (4), or anti-insulin factors in the serum (5) cannot account for the insulin tolerance. Beyer (3) has found, however, that the liver of the KL mouse is capable of inactivating much larger doses of insulin than the liver of the control mouse. This increased *in vitro* insulinase activity of the liver is not shared by the kidney, spleen, muscle or blood of the KL animal (3).

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[2] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Syracuse University.

[3] Guidance and facilities for the isotope experiments in this investigation were provided by Dr. Harold F. Diermeier, Pharmacology Department, Upstate New York Medical Center, Syracuse, New York, for which the author is sincerely grateful.

A further investigation of this reported *in vitro* insulinase activity especially as related to *in vivo* activity, seemed advisable. If there were an increased *in vivo* insulinase activity in the KL mouse, one might expect a corresponding lack of circulating endogenous insulin and consequently a high blood sugar level, simulating a diabetic condition. Or, if the blood sugar of the KL mouse were normal, one might expect an increased rate of insulin secretion by the islets of Langerhans to counterbalance the high rate of insulin destruction. On the other hand, possibly the insulinase system is activated only after large doses of exogenous insulin. If such an "emergency response" is operating in the KL mouse, one would expect a normal blood sugar level and insulin concentration in the pancreas before insulin administration.

Our results indicate that, although a slightly increased rate of insulin destruction may exist, it cannot fully explain the insulin resistance of the KL mouse. The mechanism of insulin resistance more probably resides in the ability of the KL animal to maintain a blood sugar slightly above the convulsive level for a long period of time after large doses of insulin.

MATERIALS AND METHODS

All mice were bred and maintained in air-conditioned, properly humidified animal quarters. The insulin resistant (KL) animals[4] were screened at 40 days of age with an intraperitoneal injection of 200 units of insulin (Iletin, U-500).[5] During the screening procedure, food pellets were placed on the bottom of the cage. Control mice of the BUB strain[6] were used.

Destruction I^{131}-labeled insulin by rat liver extracts: The *in vitro* insulinase activity was assayed by the method of Mirsky *et al.* (6), but the liver homogenates were prepared in 4 volumes of 0.067 *M* phosphate buffer. One ml of the supernatant fluid was incubated with 1 ml. of an insulin solution, containing 0.027 unit of zinc-insulin (Iletin) and 0.5 microcurie of insulin-I^{131},[7] in a Dubnoff shaker at 38° C. As a control, the same amount of insulin was added to 1 ml. of phosphate buffer. After 30 minutes of incubation, the mixture was poured into tubes containing 2 ml. of 10% trichloroacetic acid (TCA), and centrifuged for 10 minutes at 18,000 ×g. The supernatant was diluted to 5 ml. with TCA and the precipitate dissolved in 5 ml. of 30% KOH. The radioactivity of 2 ml. aliquots from the supernatant and dissolved precipitate was measured with an end-window Geiger tube with a window thickness of 2.5 mg./cm.^{2}. Samples were counted in duplicate for 5 minutes and appropriate corrections for background counts were made. The per cent of total counts present in the supernatant fraction was used as a measure of insulin breakdown. The radioactivity in the supernatant of the control solution represented the small amount of non-precipitable insulin-I^{131} and was subtracted from the experimental values.

In vitro insulinase activity determined by the mouse bioassay technique: The liver homogenates were prepared as above in 3 volumes of cold 0.067 *M* phosphate buffer. After centrifugation the pH was adjusted if necessary to 7.5 with 1% KOH. One ml. of the supernatant was incubated in a 37° C waterbath with 12 or 100 units of zinc-insulin (Iletin) for 30 minutes, and the mixture was shaken at 70 cycles per minute. As a control 1 ml. of insulin was incubated with 1 ml. of phosphate buffer.

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[4] Originally from the colony of Dr. Herman B. Chase, Brown University.

[5] Generous quantities of U-500 zinc-insulin were supplied by the Lilly Research Laboratories, through the courtesy of Drs. O. K. Behrens and W. R. Kirtley.

[6] Originally from the colony of Dr. J. W. Wilson, Brown University.

[7] Prepared by Abbott's Laboratories.

For the insulin assay, an aliquot of the incubation mixture was diluted so that it contained a maximum of 0.05 unit per 0.25 ml. of solution. This was injected intraperitoneally into groups of 6 female Swiss albino mice[8] which had been fasted in individual metabolism cages for 16 hours. The bioassay animals were 4 months old and weighed 22–23 gm. The per cent of the initial blood sugar at 60 minutes was used as a measure of the amount of insulin left in the incubation mixture. Control mice received the diluted insulin-buffer solution (0.05 and 0.025 unit). The bioassay mice were re-used at intervals not shorter than one week. Groups were interchanged so that animals which received KL extract the first time were injected with BUB extract the next time.

The blood sugar was measured according to the Nelson-Somogyi method (7) on small samples (0.03 ml.) of tail blood. The volumes of all solutions were reduced proportionally.

Insulin-I^{131} destruction by intact mice: The *in vivo* rate of insulin destruction was measured by the technique of Mirsky *et al.* (8). Fed mice were injected intraperitoneally with 0.25 ml. of an insulin solution containing 10 units of zinc-insulin (Iletin) and 10 microcuries of insulin-I^{131}. They were sacrificed 60 minutes later and homogenized in a Waring Blendor for 8 minutes with 70 ml. of cold 10% TCA. All the glassware was kept cold before use and 1 mg. of bovine plasma albumin was added to decrease the adsorption of I^{131} to the glass (9). Part of the mixture was centrifuged and the radioactivity determined on 2 ml. aliquots of the whole homogenate and supernatant. Conditions and technique of counting were as described above. Samples were counted for 5 minutes or until at least 2,000 counts were recorded. The per cent of total counts present in the supernatant fraction in the form of I^{131}-labeled fragments was used as an index of insulin breakdown. To account for the amount of insulin destroyed during the homogenization process, one group of mice was killed immediately after the insulin injection and the degree of insulin breakdown in these control mice was determined.

Glucose tolerance: For the glucose tolerance tests, mice were fasted 16 hours in individual metabolism cages and injected intraperitoneally with 1 mg. of glucose per kg. bodyweight. The glucose was injected as a 10% saline solution and blood sugar values determined before and at intervals after glucose administration.

Pancreatic extraction: The pancreases of mice, fasted for 15 hours, were extracted according to the technique of Best *et al.* (10). The final precipitate, consisting of insulin and the hyperglycemic factor, was dissolved in isotonic saline (pH 2.5) of 30 times the volume of the original pancreatic weight. In most cases the pancreases of 2 animals were pooled.

Bioassay of the pancreatic extracts for its insulin content was carried out on fasted BUB male and Light female mice.[9] The age, sex and body weight of the bioassay mice in the experimental and control groups was matched. The average blood sugar drop of 6 mice during 60 minutes was used as a measure of insulin activity in the extracts. Extraction and bioassay of the experimental (KL) and control (BUB) pancreases were performed simultaneously. As a control for the bioassay, fasted BUB male mice were injected intraperitoneally with 0.4 ml. of acid saline and the blood sugar change determined. To estimate the amount of insulin extracted from the KL and BUB pancreas, standard doses of insulin, dissolved in isotonic acid saline, were injected into fasted BUB male mice and the drop in blood sugar determined.

Insulin tolerance: For the insulin tolerance experiments the mice were kept in individual metabolism cages. Insulin was administered intraperitoneally and blood sugar levels were determined before and at intervals after insulin injection. All mice were allowed water and Purina Chow pellets *ad libitum* during the test.

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[8] Purchased from the Albino Farms, Red Bank, New Jersey.

[9] Kindly supplied by Dr. H. B. Chase.

RESULTS

Insulin inactivation: A preliminary determination of the *in vitro* insulinase activity with radioactive insulin was carried out in an attempt to confirm Beyer's findings of an increased insulinase activity in the KL liver (3). The results in Table 1, however, indicate that the per-cent of insulin-I^{131} breakdown by liver extracts of insulin resistant (KL) and control (BUB) mice is similar (t = 0.7; P = .52). Since a very small concentration of insulin was used in our incubation medium (0.027 unit) and since the increased insulinase activity may only manifest itself in the presence of large doses of insulin, Beyer's experimental technique was repeated, using the bioassay method to measure *in vitro* insulin braekdown. Liver extracts were incubated with 12 or 100 units of insulin for 30 minutes and the amount of active insulin remaining in the incubation mixture was determined. Figure 1 represents the per cent of the initial fasting blood sugar of Swiss mice, one hour after the intraperitoneal injection of liver-insulin mixture. The average initial fasting blood sugar value of Swiss mice is 51 ±4 mg.%. The results in Figure 1 indicate that there is an equal amount of insulin left in the experimental (KL) and control (BUB) incubation mixtures, for the blood sugar drop in both groups of bioassay mice is similar. This implies that the *in vitro* inactivation of 12 or 100 units of insulin by the KL and BUB liver is similar. Blood sugar levels of bioassay mice injected with standard doses of 0.05 and 0.025 units of insulin (Fig. 1) indicate that approximately 50% of the insulin substrate is broken down during the 30 minute incubation period. We can conclude therefore, that the livers of insulin resistant (KL) and control (BUB) mice inactivate insulin *in vitro* to the same extent.

TABLE 1. PRELIMINARY DETERMINATION OF THE *in vitro* LIVER INSULINASE ACTIVITY OF INSULIN RESISTANT (KL) AND CONTROL (BUB) MICE

Strain	Sex	No.	Age (mo.)	Body wt. (gm.)	Liver wt. (gm.)	Av. counts/min. /aliquot		% insulin break-down	Insulinase activity	Recovery (%)
						Pre-cipitate	Super-natant			
KL	M	3	2–4	30	1.9	258	610	70	45 ±0.9	94
BUB	M	3	2–4	29	1.7	277	617	69	44 ±1.0	96
Control solution						674	223	25		

Since *in vitro* results may not accurately reflect *in vivo* conditions, the rate of insulin-I^{131} breakdown in intact animals has been determined. Intact insulin resistant (KL) mice do inactivate 10 units of radioactive insulin at

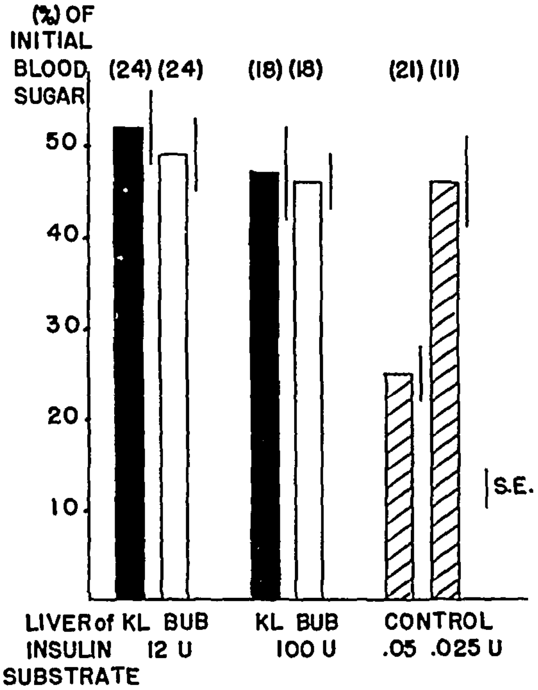

FIG. 1. *In vitro* insulinase activity of insulin resistant (KL) and control (BUB) liver, incubated with 12 and 100 units of insulin for 30 minutes at 38° C. ( ) indicates the number of bioassay mice.

a slightly higher rate than control (BUB) animals, but the difference is not significant at the one per cent level (t = 2.3; P = .0344) (Table 2). Increased rate of insulin destruction therefore cannot fully explain the insulin resistance in the KL mouse.

Glucose tolerance and pancreatic extraction: The fasting blood sugar levels and glucose tolerance tests of insulin resistant (KL) mice are similar to those of the control (BUB) strain (Fig. 2). If the degree of insulin destruction in the KL mouse were high, a corresponding increase in the rate of insulin secretion might be expected in order to account for the normal blood sugar levels. To test this possibility, pancreases of insulin resistant (KL) mice were extracted and the insulin content was determined by the bioassay technique. Figure 3 represents the blood sugar levels of BUB and Light bioassay mice before and one hour after the intraperitoneal injection of KL and BUB pancreatic extracts. The decreases in blood sugar

TABLE 2. INSULIN-I^{131} BREAKDOWN IN INTACT INSULIN RESISTANT (KL) AND CONTROL (BUB) MICE AFTER THE INTRAPERITONEAL INJECTION OF 10 UNITS OF INSULIN, CONTAINING 10 MICROCURIES OF INSULIN-I^{131}

Strain	No.	Sex	Age (mo.)	Body wt. (gm.)	Av. counts/min. /total sample		% insulin breakdown	Insulinase activity
					Homogenate	Supernatant		
KL	10	M	2–3	30	47,030	9,893	23.7	21.1 ± 1.6
BUB	9	M	2–3	30	42,923	7,999	18.6	17.1 ± 1.4
Control (KL+BUB)	3	M	2–3	30	48,376	777	1.6	

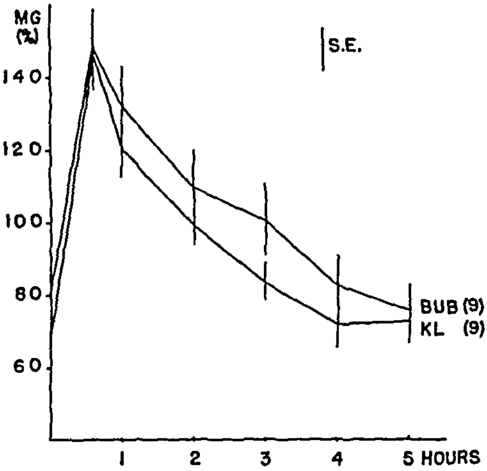

FIG. 2. Glucose tolerance of insulin resistant (KL) and control (BUB) mice. ( ) indicates the number of mice.

levels are the same and consequently the amount of insulin in the experimental (KL) and control (BUB) pancreatic extracts must be similar. By comparing the blood sugar drop after injection of pancreatic extracts with the blood sugar decline after known doses of insulin, it is apparent that 0.4 ml. of pancreatic extract contains approximately 0.01 unit of insulin. Since the dilution factor in this technique is 30, it appears that the pancreas of both the KL and BUB mouse contains approximately 0.75 unit of insulin per gram of tissue. This corresponds to the fasting values reported by Marks *et al.* (11).

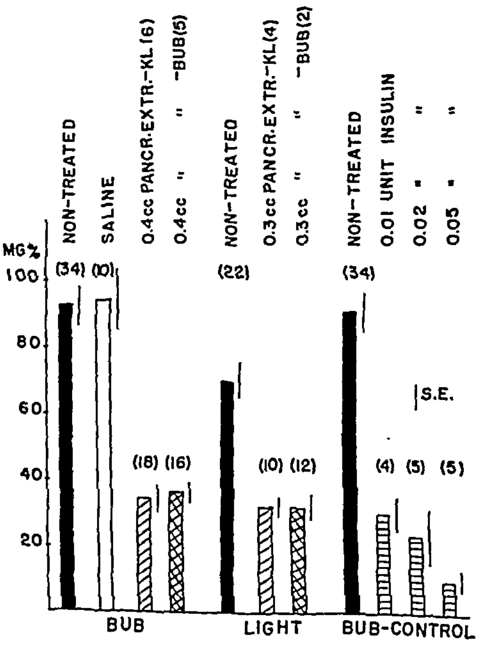

FIG. 3. Extractable insulin from the pancreas of insulin resistant (KL) and control (BUB) mice. Values represent blood sugar levels of fasted mice before and one hour after the intraperitoneal injection of the pancreatic extracts. ( ) indicates the number of mice.

Insulin tolerance curve: Insulin resistance could be due to a failure of insulin to exert its usual blood sugar lowering effect. That this is not the case in the KL mouse is indicated in Figure 4. The injection of one unit of insulin into fed KL mice is followed by a drop in blood sugar level, with a gradual return to normal in 4 hours. This blood sugar response is similar to that of control (BUB) mice (Fig. 4). After 200 units of insulin, however, the blood sugar in the KL mouse declines at about the same rate as after one unit, but remains at approximately a 30 mg.% level for 20 hours. Not until 27 hours after the insulin injection does the blood sugar level return to normal (Fig. 4). The blood sugar of the control BUB mice decreases rapidly and the animals convulse in 2 hours. These experiments indicate that insulin does have a blood sugar lowering effect in the KL mouse and that the insulin resistance mechanism probably resides in the ability of the KL animal to maintain a blood sugar slightly above the convulsive level for a long period of time.

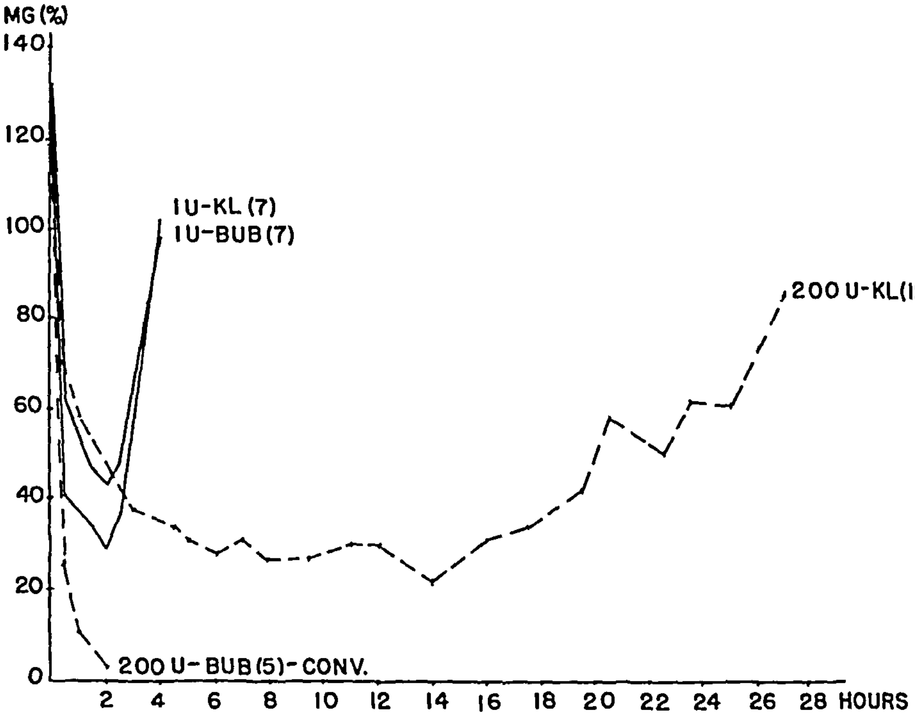

FIG. 4. Insulin tolerance of fed insulin resistant (KL) and control (BUB) mice after the intraperitoneal injection of 1 and 200 units of insulin. ( ) indicates the number of mice.

DISCUSSION

The results reported here indicate that an increased rate of insulin destruction cannot fully explain the insulin resistance of the KL mouse, as is evident from the studies on insulin breakdown *in vitro* and *in vivo*. Using both the bioassay and the insulin-I^{131} technique, to assay insulinase activity, it is clear that no difference exists in the rate of insulin breakdown between the experimental (KL) and control (BUB) groups of mice.

Indirect evidence for a normal rate of insulin degradation in the insulin resistant (KL) mouse is provided by the normal blood sugar values and pancreatic insulin content. If the rate of insulin breakdown were high, a correspondingly high blood sugar level or an increased rate of pancreatic insulin secretion would be expected to counterbalance the rapid rate of insulin destruction. As pointed out before, a normal blood sugar value, accompanied by a normal insulin concentration in the pancreas could also be interpreted in terms of an "emergency response." The insulin resistant (KL) mouse may increase its insulinase activity only after the administration of large doses of insulin. In that case, the untreated mouse would have a normal rate of insulin destruction and secretion, keeping the blood sugar balanced.

The emergency response, however, is unlikely in the light of observations of the blood sugar response of the KL mice after 200 units of insulin. Under these conditions, the blood sugar level declines to a value of 30 mg.% and remains at this approximate level for about 27 hours. If the rate of insulin breakdown in the KL mouse were high, or if the injection of 200 units of insulin would activate the *in vivo* insulinase system, the blood sugar level would be expected to return to normal values much earlier than is actually the case.

We feel that the mechanism of insulin resistance resides in the ability of the KL mouse to maintain a blood sugar slightly above the convulsive level for a long time after insulin administration, in contrast to a control mouse which allows the blood sugar to drop rapidly and reach the convulsive level. This result implies that the insulin resistant mice have access to a readily available carbohydrate source which they can utilize to counterbalance the insulin-induced hypoglycemia. This source does not necessarily have to be extensive since the animal maintains the blood sugar at only 20 mg.% above the convulsive level. Evidence for the contributing role of liver glycogen stores, food intake and intestinal absorption to the insulin resistance phenomenon in the KL mouse will be presented in a future report.

Acknowledgments

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AN EFFECT OF CORTICOTROPIN ON UREA FORMATION IN ADRENALECTOMIZED-NEPHRECTOMIZED RATS[1]

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ABSTRACT

Corticotropin has been found to share with growth hormone the capacity to diminish the accumulation of blood urea nitrogen consequent to the intravenous infusion of an amino acid solution (protein hydrolysate) into acutely adrenalectomized nephrectomized rats maintained with hydrocortisone. Milligram for milligram ACTH is as active as growth hormone. The nitrogen retaining action of ACTH is abolished by boiling in 0.1N NaOH but not by more gentle treatment with NaOH. ACTH activity is destroyed by oxidation with hydrogen peroxide but can be restored by incubation with cysteine, in keeping with other adrenal and extra-adrenal activities of ACTH. Growth hormone activity is not definitely destroyed by H_2O_2 treatment. It is concluded that the above action of ACTH on nitrogen metabolism is another extra-adrenal action of ACTH which is shared with growth hormone but cannot be accounted for by contamination of the ACTH with growth hormone, or vice versa.

RUSSELL and Capiello have reported that the administration to nephrectomized rats of both partially and highly purified preparations of growth hormone results in a significant decrease in the rate of urea formation following the intravenous infusion of protein hydrolysates (1, 2). In view of the recent claims from this laboratory and elsewhere that highly purified corticotropin exerts a number of metabolic actions in adrenalectomized animals which simulate those of growth hormone (3, 4, 5, 6, 7, 8, 9) an investigation of the influence of corticotropin on nitrogen metabolism was initiated using a modification of Russell's technique. At our instigation Russell has already tested a sample of oxycel ACTH and has reported that it does indeed have a growth hormone-like effect in the nephrectomized rat (10). The present study confirms and extends Russell's observation by demonstrating the effects of both growth hormone and ACTH in adrenalectomized-nephrectomized rats, thereby establishing the extra-adrenal nature of the response to the two hormones.

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MATERIALS AND METHODS

Twenty-four hour fasted male rats weighing from 200 to 250 grams were used. They were anesthetized with pentobarbital. Nephrectomy and adrenalectomy were carried out in one stage, following which the rats received 1 mg. of hydrocortisone hemisuccinate intraperitoneally. The rats were then placed in a chick incubator at 33° C to maintain body temperature and were kept there throughout the experiment except for blood sampling. Two hours postoperatively the test hormone was injected intraperitoneally, the controls receiving a corresponding volume of saline or 2 mg. of human serum albumin. Two hours later a tail blood sample was obtained, the animals were reanesthetized and 1 ml. of an amino acid solution (Parenamine) containing 15 mg. N was injected into the saphenous vein for each 100 gm. body weight. Two hours after this a second blood sample was obtained. The blood was analyzed for urea N by the method of Archibald *et al.* (11).

The characteristics of the hormones used and the methods of inactivation have been described in previous reports (5, 8). They included oxycel ACTH from the Wilson Company (lots 102201, 103322), α_1-α_2 ACTH from the American Cyanamid Co. and growth hormone from the Endocrinology Study Section of the National Institutes of Health (BGH-1).

RESULTS

Two sets of experiments were done, one in the summer of 1957 and the other in the spring of 1958. It will be noted in Table 1 that although qualitatively the results were comparable, quantitatively there was some variation between the response to the same hormone in 1957 and 1958. The table records the increase in urea nitrogen over the two hour period beginning with the amino acid infusion. In confirmation of Russell and Capiello growth hormone was found to lower significantly the urea accumulation rate compared to that of untreated controls. In the 1957 series the response was quite variable as indicated by the high standard error of the mean. The 1958 group was more consistent although the response to 1 mg./100 gm. was relatively small and was not increased when the dose was doubled.

Three samples of ACTH were tested. In 1957, 1 mg. of oxycel ACTH and 0.5 mg. of α_1-α_2 ACTH per 100 gm. body weight brought about depressions in urea formation which were of the same order of magnitude as has been found with growth hormone. These results were confirmed in the 1958 series with a different lot of oxycel ACTH. In this case the response was greater than to the corresponding dose of growth hormone.

We have previously reported that the extra-adrenal metabolic effects of ACTH are lost in parallel with its adrenal stimulating activity when the hormone is boiled in 0.1N NaOH or oxidized with H_2O_2 (5, 6, 7, 8). In the latter case activity may be restored in part at least by treatment of the oxidized hormone with cysteine. Growth hormone activity is destroyed by mild treatment with alkali (0.1N NaOH for 24 hours at 26° C) (10) but ACTH resists this treatment (5). The results of comparable studies measuring urea accumulation are found in Table 1. Oxycel ACTH boiled in 0.1N alkali lost activity; when subjected to the more gentle alkali treat-

TABLE 1. INFLUENCE OF GROWTH HORMONE AND ACTH ON RISE IN BLOOD UREA N DURING 2 HOURS FOLLOWING INTRAVENOUS INFUSION OF 15 MG. OF AMINO ACID N/100 GMS. BODY WEIGHT IN ADRENALECTOMIZED-NEPHRECTOMIZED RATS

Experimental groups	No. of rats	Rise in urea N in 2 hrs. mg.% (Mean ±S.E.)	P vs. control
Control—1957	21	20.42 ±1.05	—
G.H. 1 mg./100 gm.	6	11.82 ±2.25	<.01
G.H.-H_2O_2 treated	7	14.89 ±1.12	<.01
Oxycel ACTH 1 mg./100 gm.	13	13.09 ±1.02	<.01
Oxycel ACTH 0.1N NaOH, 26° C, 24′	7	15.80 ±0.88	<.01
Oxycel ACTH 0.1N NaOH, 100° C, 20″	7	18.02 ±2.25	N.S.
α_1-α_2 ACTH, 0.5 mg./100 gm.	6	13.49 ±1.16	<.01
α_1-α_2 ACTH, 0.75 mg./100 gm., H_2O_2 treated	6	17.61 ±1.31	N.S.
α_1-α_2 ACTH, 0.75 mg./100 gm., H_2O_2-cysteine	6	16.59 ±0.92	N.S.
Control—1958	34	20.19 ±0.55	—
G.H. 1 mg./100 gm.	9	17.14 ±0.54	<.01
G.H. 2 mg./100 gm.	7	17.10 ±0.76	<.01
G.H. 2 mg./100 gm., H_2O_2 treated	11	18.64 ±0.99	N.S.
Oxycel ACTH—1 mg./100 gm.	15	15.69 ±0.48	<.01
Oxycel ACTH—1 mg./100 gm., 0.1N NaOH, 100°, 20″	6	18.35 ±1.10	N.S.
Oxycel ACTH—H_2O_2 treated	13	19.58 ±0.85	N.S.
Oxycel ACTH—H_2O_2-cysteine	13	17.65 ±0.72	<.02
Oxycel ACTH—2 mg./100 gm.	7	15.03 ±0.68	<.01
Oxycel ACTH—H_2O_2 treated	9	20.77 ±0.99	N.S.
Oxycel ACTH—H_2O_2-cysteine	8	14.28 ±0.85	<.01

ment it retained its effect. Hydrogen peroxide treated ACTH was invariably inactive. Reactivation of the α_1-α_2 ACTH (0.75 mg./100 gm.) was not accomplished in 1957. On the other hand in the 1958 series some reactivation of oxycel ACTH was achieved at the 1 mg./100 gm. dose and unequivocal reactivation at the 2 mg./100 gm. dose. In the former case the urea rise of 17.65 ±0.72 mg.% in the cysteine treated group was significantly less than the control ($P < .02$), but still not significantly less than the H_2O_2 treated hormone (19.58 ±0.48 mg.%). We have previously found in other studies that cysteine reactivation may not be complete and hence that it is necessary to use maximal rather than minimal doses to demonstrate the phenomenon (5, 7, 8). In contrast to the results with ACTH, treatment of growth hormone with H_2O_2 did not lead to clearcut inactivation. In the 1957 series the H_2O_2 treated growth hormone was clearly active. In the 1958 series the results were indecisive since the urea rise from the H_2O_2 treated hormone was not significantly different from either the untreated hormone or the control. However, it should be noted that in contrast to the ACTH effects, the response to growth hormone was small to begin with and hence it is difficult to compare effects of various treatments of the hormone.

DISCUSSION

These results confirm the preliminary report of Russell of a nitrogen retaining effect of ACTH in nephrectomized rats which is comparable to that reported by her for highly purified growth hormone (2). In the present report the extra-adrenal nature of the response is established by the use of hydrocortisone maintained acutely adrenalectomized rats. Milligram for milligram ACTH is at least as active if not more active than growth hormone, although as emphasized in previous papers, the doses of ACTH used are huge when expressed in units. The response of ACTH to various inactivation and reactivation procedures corresponds with what we have reported for other adrenal and extra-adrenal actions of ACTH (5, 6, 7, 8). In view of the dose relationships and the fact that growth hormone was not clearly inactivated by H_2O_2 treatment whereas ACTH was, one can definitely rule out the possibility that the response to the ACTH samples used was due to contamination with growth hormone. It might be noted in passing, too, that we have found H_2O_2 treated growth hormone to be fully active when tested by its ability to induce a positive nitrogen balance in tube-fed rats (13). Conversely, since growth hormone is inactivated by mild alkali treatment (10, 12) whereas ACTH is not, the very unlikely possibility that the growth hormone effects were due to ACTH contamination need not be seriously considered. The same reasoning also makes mutual contamination of both hormones by a common third factor rather unlikely.

These results can be taken as additional evidence that ACTH shares certain activities with growth hormone at least when the former hormone is administered in very large doses. The physiological significance, if any, of the activity described in this report is as yet unclear. Attempts to induce positive nitrogen balance in tube-fed adrenalectomized rats by the administration of ACTH have so far been unsuccessful, although growth hormone, as expected, is quite active. Further investigation of possible growth promoting effects of ACTH in the absence of the adrenal gland is clearly indicated.

Acknowledgments

We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, Illinois for the oxycel ACTH, to Dr. Paul Bell of Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y. for α_1-α_2 ACTH, to the Endocrinology Study Section of the National Institutes of Health for the growth hormone, and to Dr. A. Scribner, the Winthrop Laboratories, New York, N. Y. for the Parenamine.

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EFFECT OF FREQUENT HOUSING CHANGES AND OF MUSCULAR EXERCISE ON THE THYROID GLAND OF MICE[1]

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ABSTRACT

The possibility that the psychological stimulation resulting from frequent changes of housing might have an effect on the thyroid was investigated in mice "transferred" daily to different types of cages for a period of 8 weeks. Similarly, the effect of forced muscular exercise was investigated in mice made to walk daily 3½ to 4 hours in a rotating cage for a period of 8 weeks. All animals were fed controlled diets with low or adequate levels of dietary iodine.

Control mice on the low iodine diet had enlarged thyroids showing histological signs of stimulation. "Transferred" or exercised mice fed the same diet failed to show thyroid stimulation. Thus, frequent housing changes and forced muscular exercise prevented the thyroid stimulation resulting from iodine deficiency. This depressing effect on the thyroid does not appear to be due to unspecific stress, since adrenal weight was not altered.

IT IS widely held that human hyperthyroidism may arise from acute or chronic psychological stimulation (1, 2). However, acute psychological stimulation was shown by Brown-Grant *et al.* (3, 4) to produce an immediate decrease in the rate of release of radioiodine from the thyroids of immobilized rabbits. Such a decrease may be the result of associated stress since it is known that stress depresses the activity of the thyroid gland (5). Therefore, an experiment was designed to investigate the effect of psychological stimulation on the thyroid, using a stimulus mild enough to avoid stress. Another stimulus, namely forced muscular exercise, has also been reported to depress thyroid activity (5). However, this effect was slight as compared with that of other stressing agents which produced an intense depression of the thyroid gland. It was possible that forced muscular exercise induced two antagonistic effects masking each other: a thyroid depression due to stress, and a stimulation of the gland attributable to an increased consumption of thyroid hormone as suggested by the rapid disappearance of injected thyroxine from the blood (6). To investigate whether or not such a stimulation takes place, mice were subjected to exercise mild enough to avoid stress.

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METHODS AND RESULTS

Psychological stimulation

A mild stimulus acting over a long period of time may have a definite effect on the thyroid gland, provided it can repeatedly elicit a condition of alertness in spite of frequent repetition over the experimental period. The procedure adopted consisted of providing mice with a new environment each day, by transferring them to different types of cages. Furthermore, in the experience of this laboratory, a change in iodine metabolism is more likely to result in a change of thyroid activity if the iodine intake of the experimental animals is critical. Accordingly, control and experimental animals were subdivided into two groups, given either a low or adequate amount of dietary iodine.

C_3H mice were transferred through 8 cages of various shapes. Since transfers took place once a day, the mice were returned to the same cage every 8 days. Control mice were kept in the same suspended cage throughout the experiment, but they were handled daily. All the animals were housed in groups of 6. Temperature (80.0 ± 5°F) and lighting (12 hours daily) were automatically controlled. Twenty-four mice, 4–5 weeks old, were fed a low iodine diet consisting of 78.85% cornmeal, 10% yeast, 1% calcium carbonate and 0.15% sodium chloride, and containing 3 μg. iodine per 100 gm., while an equal number of animals received the same diet supplemented with sodium iodide to raise the iodine level to 10 μg. per 100 gm. These 2 groups were divided into subgroups of 12 control and 12 test mice.

After 8 weeks, the animals were sacrificed with ether. One thyroid lobe was dissected free and weighed on an analytical balance. The other lobe was fixed in Bouin's fluid, sections were cut at 6 μ, and stained with Masson's trichrome. Thyroid cell height for each animal was measured in 80 cells taken at random. The percentage of follicles depleted of colloid was estimated by examining all follicles in one thyroid section from each animal. The adrenals were weighed after fixation.

In the iodine deficient mice the controls had enlarged thyroids (2.8 mg./lobe) while the transferred mice had the same thyroid weight (0.8 mg./lobe) as the iodine supplemented mice (0.7 mg./lobe) (Table 1). The thyroids of mice submitted to a daily change of cage (Table 1) had lower cells and fewer follicles depleted of colloid than those of corresponding control animals regardless of the dietary iodine level. Adrenal weights were identical in transferred and control groups: 2.8 mg. under conditions of low iodine intake and 2.7 mg. with an adequate iodine intake. No changes in body weight were observed in the mice receiving the low iodine diet, while the transferred mice receiving adequate dietary iodine weighed less than their controls (Table 1).

Muscular exercise

To investigate the influence of muscular exercise on thyroid morphology, the mice were placed in a motor driven rotating cage, 3½–4 hours per day, during 8 weeks. The cage had a circumference of 65 cm. and turned at 4½ revolutions per minute, thus enforcing a walking pace. Controls were handled daily. Other conditions were identical with those of the experiment described above.

In the animals fed on the low iodine diet, the exercised mice had a lower thyroid weight, a decreased cell height, and fewer follicles depleted of colloid (Table 2) than the non-exercised controls. In the animals receiving the diet supplemented with iodine, exercise had no significant effect on the

Table 1. The effect of daily transfer of cage on the mouse thyroid

μg. iodine per 100 gm. diet	Treatment	No. of mice	Body weight, gm.		Left thyroid lobe weight, mg.		Epithelial cell height, μ		% follicles depleted of colloid	
			Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*
3	Controls	9	24.2±1.12	0.8 −0.7	2.8±0.2	<0.001	16.8±0.3	<0.001	91.3±1.9	<0.001
	"Transferred"	8	24.6±0.24		0.8±0.2		9.6±0.3		4.8±0.8	
10	Controls	9	25.4±0.21	0.02−0.01	0.7±0.1	0.5−0.4	11.7±0.3	<0.001	6.0±1.0	0.3−0.2
	"Transferred"	8	23.3±0.21		0.6±0.1		9.3±0.3		4.2±1.3	

* "t" test.

Table 2. The effect of mild daily muscular exercise on the mouse thyroid

μg. iodine per 100 gm.-diet	Treatment	No. of mice	Body weight, gm.		Left thyroid lobe weight, mg.		Epithelial cell height, μ		% follicles depleted of colloid	
			Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*
3	Controls	7	21.4±0.26	0.1−0.05	2.8±0.4	0.1−0.05	15.4±0.5	<0.001	94.6± 1.7	<0.001
	Exercised	10	23.4±0.18		1.5±0.5		11.3±0.7		20.3±11.6	
10	Controls	8	24.5±0.25	0.8±0.7	0.9±0.2	0.4±0.3	9.7±0.3	0.2-0.1	4.7± 0.8	0.8−0.7
	Exercised	10	24.9±0.29		1.3±0.2		10.4±0.2		4.9± 1.1	

* "t" test

thyroid gland (Table 2). Adrenal weights were not altered by exercise: 2.7 mg. in both groups of mice receiving the low iodine diet, and 2.3 mg. in both groups receiving the diet supplemented with iodine. No significant changes in body weight were observed.

DISCUSSION

The effect of psychological stimulation and of muscular exercise on the thyroid gland reported in the literature (3, 5) may possibly be due to stress associated with the experimental procedure. The present experiments were an attempt to investigate these stimuli, using procedures avoiding stress.

In fact, the stimuli selected, namely daily changes of housing and mild daily exercise, did not produce the increase in adrenal weight and the decrease in body weight usually associated with prolonged stress, except in one case (Table 1); but in this particular case the thyroid weight and the proportion of follicles depleted of colloid were not changed (Table 1). Thus, it is likely that the lower level of thyroid activity observed in this work is not due to stress.

The possibility that the lower thyroid activity in the stimulated mice be due to a higher iodine intake resulting from increase in food consumption appears very unlikely. While the exercised mice may have ingested more food, the schedule required to refill the food jars suggests that the transferred mice ate not more, and perhaps even less than controls. In addition, an increased food consumption would result in increased metabolism and presumably a higher thyroid hormone consumption. A stimulation of the thyroid would follow, which would counteract the inhibition due to the higher iodine intake.

Thus, it is probable that the thyroid inhibition was produced by the stimuli according to a specific mechanism, perhaps mediated through the hypothalamus (7) and resulting in an inhibition of thyrotrophic hormone secretion.

The finding that psychological stimulation may inhibit the thyroid gland not only for a short time (3), but also over long periods of time (Table 1), fails to substantiate the hypothesis that simple chronic psychological overstimulation is a cause of thyroid hyperfunction, and suggests rather that it may lead to a hypofunction of the gland.

The observation that muscular exercise in the absence of stress also yields evidence of thyroid inhibition does not support the hypothesis that this stimulus had an activating effect on the thyroid gland (5), as might have been expected from the low level of circulating thyroid hormone in the exercised rat (6). On the contrary, muscular exercise prevented the thyroid stimulation in response to iodine deficiency (Table 2), as in the case of chronic psychological stimulation. In fact, during their daily exercise, the mice were in a condition of alertness which may not differ much from that of mice transferred to new cages.

Acknowledgment

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POTENTIATION OF BIOLOGICAL ACTIVITIES OF STEROIDS BY CARCINOGENIC HYDROCARBONS

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ABSTRACT

Recent studies by Huggins and Pollice (*J. Exp. Med.*, Jan. 1958) showed that a mammary fibroadenoma of rats, refractory to steroid therapy alone, responded in a remarkable fashion to combined administration of 3-methylcholanthrene and androstan-17β-ol-3-one. These results suggested that administration of carcinogenic hydrocarbons may alter sensitivity of certain cells to steroid hormones.

Oral administration of 3-methylcholanthrene, 2-anthramine, benz-α-pyrene, and 7-methylbenzanthracene, all carcinogenic agents, markedly potentiated both the androgenic and myotrophic effects of 17-methyltestosterone. Noncarcinogenic hydrocarbons investigated were nearly inactive in this regard. Oral and direct administration of 3-methylcholanthrene potentiated both the glycogenic and antiphlogistic properties of hydrocortisone. Inactive hydrocarbons, anthracene and phenanthrene, failed to induce this type of phenomenon.

Both *in vivo* and *in vitro* studies indicate that the ability of carcinogenic hydrocarbons to potentiate biological effects of steroids is due to a direct effect of carcinogenic agent at the cellular level and not to a decreased rate of steroid metabolism by the liver.

PREVIOUS studies by other investigators (1, 2) have suggested that local implantation of certain carcinogenic hydrocarbons antagonizes or inhibits the biological response of specific target organs to androgens, estrogens, and progestational agents. Huggins and Pollice (3) recently reported that an experimental mammary fibroadenoma, refractory to steroid therapy alone, showed a marked retardation of growth when androstane-17β-ol-3-one and 3-methylcholanthrene were administered together. When administered alone, neither compound induced a marked degree of inhibition. These results led to an hypothesis that carcinogenic agents may alter the response of certain cells to steroid hormones and that systemic administration of carcinogenic hydrocarbons may produce different effects from those observed following local implantation. The experiments described here were designed to determine the influence of carcinogenic hydrocarbons on biological effects produced by androgens and andrenocortical steroids.

METHODS

The glycogenic effect of hydrocortisone was determined by analysis of liver glycogen deposition in fasted, adrenalectomized rats (4). The seminal vesicle weight of the castrate

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rat was used as an index of androgenic activity of steroids and the levator ani muscle weight as an index of myotrophic activity (5, 6, 7). The ability of hydrocortisone to inhibit inflammation was determined by use of the croton oil-induced granuloma pouch (8). Quantitative methods for determination of steroid metabolism rates by isolated microsome-supernatant fractions of rat liver have been previously described (9). Protein determinations on isolated microsome-supernatant fractions of rat liver were performed according to previously described methods (10). Steroids and carcinogenic agents were administered subcutaneously in 0.2 ml. of carboxymethylcellulose (CMC) or orally as finely dispersed suspensions in 0.5 ml. of cottonseed oil. Direct injection of 3-methylcholanthrene into the granuloma pouch was made by first solubilizing the desired amount of material in 0.2 cc. of 50% ethanol:cottonseed oil. This material was injected into the pouch only if it was found to be in solution. Intact animals received tap water and Purina laboratory chow *ad libitum*. Adrenalectomized rats received 0.9% NaCl in addition to the stock diet.

RESULTS

1. *Potentiation by 3-Methylcholanthrene of Glycogenic and Antiphlogistic Activities of Hydrocortisone*

When hydrocortisone was administered subcutaneously in increasing amounts, and 3-methylcholanthrene orally, it was evident that the carcinogenic agent caused a significant increase in the glycogenic activity of this hormone (Fig. 1). A greater response was elicited in the presence of car-

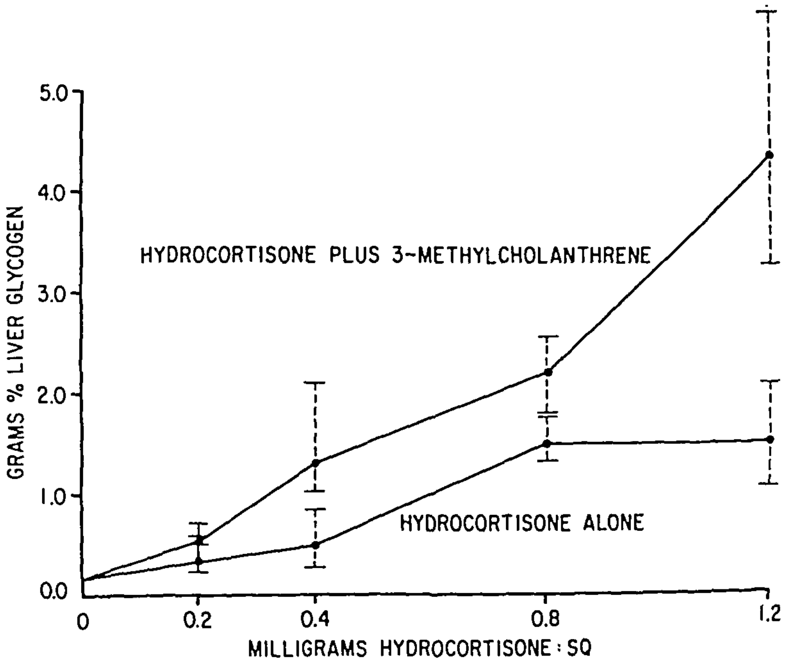

FIG. 1. Effect of oral administration of 3-methylcholanthrene on liver glycogen deposition activity of hydrocortisone. *Conditions:* One week post-adrenalectomized male rats. 3-Methylcholanthrene (2 mg.) administered orally for 5 days. On day 4 hydrocortisone administered subcutaneously at beginning of 24 hour fast; administered again on day 5 with 3-methylcholanthrene. Livers removed 7 hours later. Each point is the mean of 5 rats and the bars indicate variation from the mean.

cinogenic hydrocarbon at doses of hydrocortisone (0.8–1.2 mg.) where the liver glycogen response begins to *plateau*. That is, in the presence of 3-methylcholanthrene, the effect of hydrocortisone is greater than can be produced by the administration of additional hormone. Anthracene and phenanthrene, hydrocarbons inactive as carcinogenic agents, administered under similar circumstances, failed to produce potentiation of the glycogenic effects of hydrocortisone (Table 1).

Potentiation of glycogenic effects of hydrocortisone could result either from a direct action of carcinogenic agent on cells or tissues where steroids exert their metabolic effects, or it may result from a decreased ability of the liver to metabolize steroids. It has been demonstrated previously (9) that glycogenic activity of certain synthetic steroids is inversely related to their rates of metabolism by the liver. Therefore, if oral administration of 3-methylcholanthrene prevented inactivation of hydrocortisone, it might be reflected by an apparent increase in biological activity of the latter.

TABLE 1. EFFECT OF ORAL ADMINISTRATION OF PHENANTHRENE AND ANTHRACENE ON GLYCOGENIC ACTIVITY OF HYDROCORTISONE

Compound	Amount	Route	No. animals	Grams % liver glycogen
A. Hydrocortisone	1.2 mg.	SC	5	2.60
B. Phenanthrene	4.0 mg.	Oral	5	0.13
C. Anthracene	4.0 mg.	Oral	5	0.16
D. Untreated	—	—	10	0.22
E. A+C	—	—	5	2.44
F. A+B	—	—	5	2.53

Conditions: One-week post-adrenalectomized rats. Phenanthrene and anthracene (4.0 mg.) administered orally for 5 days. On day 4 hydrocortisone administered SC at beginning of 24-hour fast; administered again 24 hours later, along with anthracene and phenanthrene. Livers removed for analysis of liver glycogen 7 hours later.

When varying amounts of 3-methylcholanthrene were added to isolated microsome-supernatant fractions of rat liver, it was found to be inactive with regard to its ability to influence the rate of hydrocortisone metabolism (Fig. 2). Under similar circumstances, direct addition of testosterone decreased the ability of liver enzyme systems to metabolize hydrocortisone. These latter observations will be the subject of a later report on *in vivo* and *in vitro* steroid hormone interrelationships (11).

Oral administration of 2 mg. of 3-methylcholanthrene daily for seven days, followed by removal of livers and analysis of hydrocortisone metabolism rates, indicated that 3-methylcholanthrene-treated livers actually metabolized hydrocortisone at faster rates than livers from pair-fed control animals (Fig. 3).

Attention was directed to a consideration of the ability of 3-methylcholanthrene to potentiate the antiphlogistic properties of hydrocortisone using croton oil inflammation as an end-point (8). Both the carcinogen

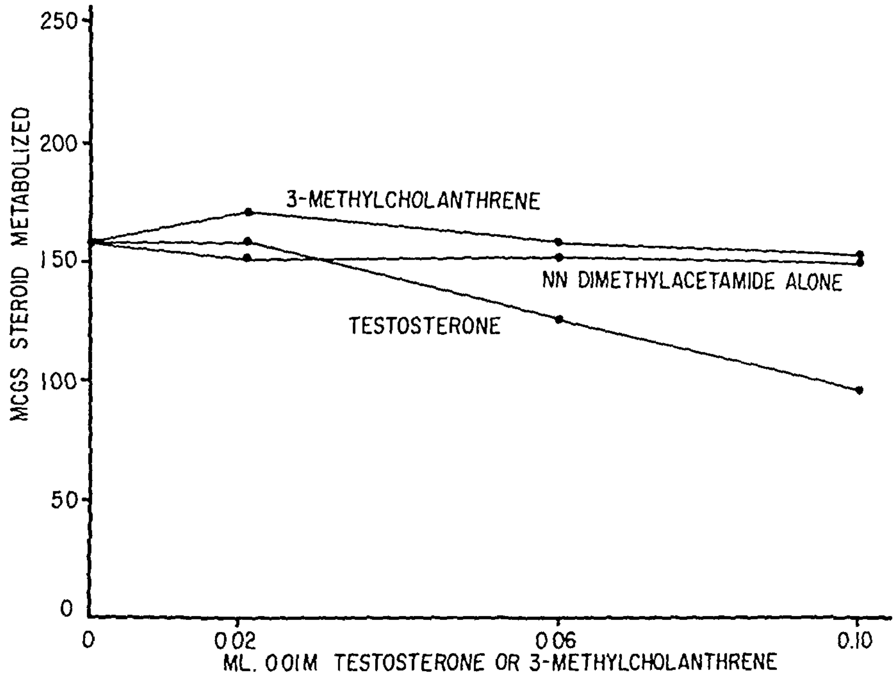

FIG. 2. Effect of testosterone and 3-methylcholanthrene on rate of reduction of hydrocortisone at C-20 ketone position by rat liver microsome-supernatant fractions. *Conditions:* Described in previous reports (9). Steroids and 3-methylcholanthrene dissolved in N′N′-dimethylacetamide.

and hydrocortisone were injected directly into the granuloma pouch. Results of these experiments (Figs. 4 and 5) show that direct administration of 3-methylcholanthrene into the granuloma pouch significantly enhances the anti-inflammatory response to hydrocortisone in both intact and adrenalectomized rats.

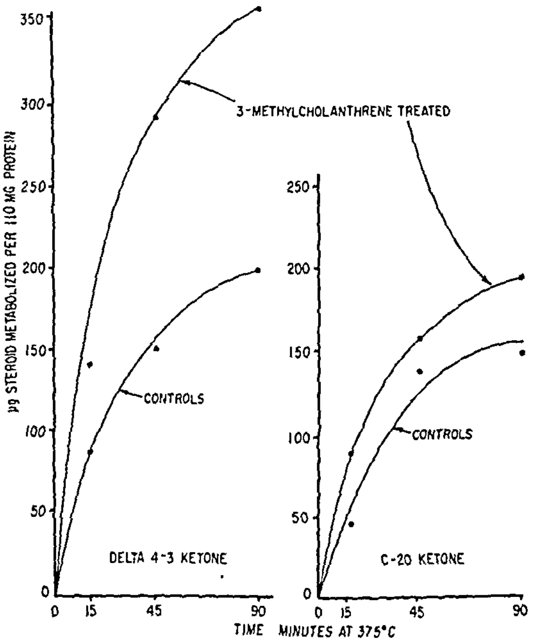

FIG. 3. Comparison 3-methylcholanthrene treated liver versus untreated liver on metabolism of hydrocortisone by isolated liver enzyme systems. Each point is the mean of duplicate determinations on duplicate samples from livers of 3 rats.

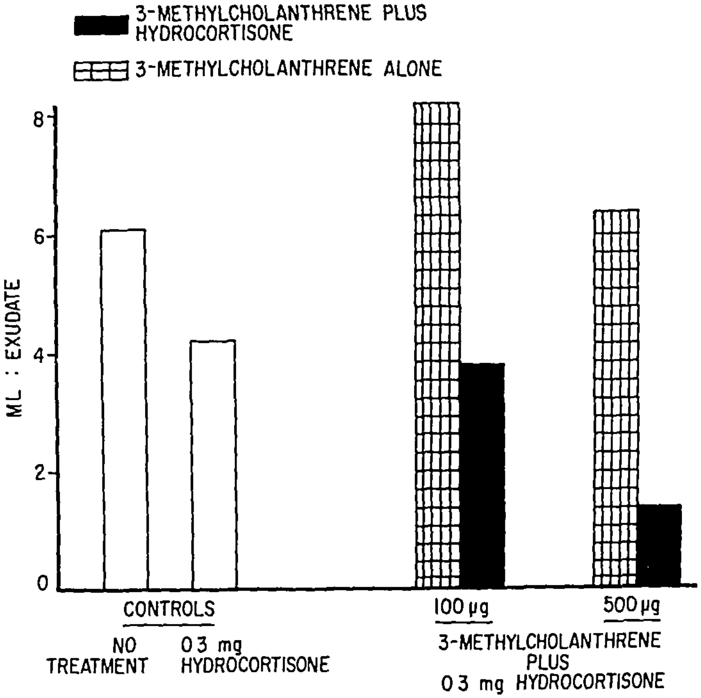

FIG. 4. Local potentiation of hydrocortisone by 3-methylcholanthrene on the granuloma pouch of Adrenalectomized rats. *Conditions:* 25 ml. air and 0.5 ml. 1% croton oil into pouch on day 1. Hydrocortisone and 3-methylcholanthrene administration started on day 2. Animals autopsied on day 7. Each bar is the mean of 5 rats.

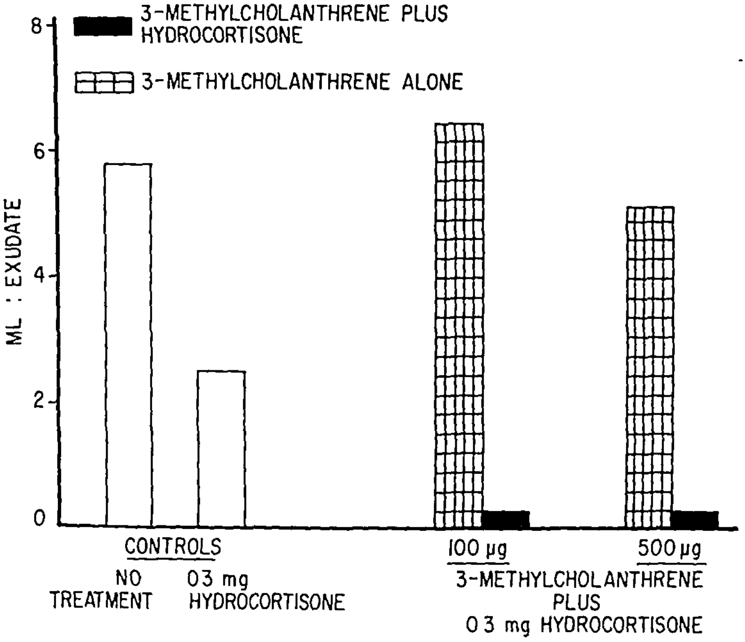

FIG. 5. Local potentiation of hydrocortisone by 3-methylcholanthrene on the granuloma pouch of intact rats. *Conditions:* 25 ml. air and 0.5 ml. 1% croton oil into pouch on day 1. Hydrocortisone and 3-methylcholanthrene administration started on day 2. Animals autopsied on day 7. Each bar is the mean of 5 rats.

2. *Effects of Various Carcinogenic Hydrocarbons on Androgenic and Myotrophic Activities of 17-Methyltestosterone*

When 3-methylcholanthrene and 17-methyltestosterone were administered orally to castrate rats, the carcinogen caused a marked potentiation of androgenic effects of the latter (Fig. 6). Relatively small amounts of 3-methylcholanthrene (0.5 mg./day) produced a maximum response.

Oral administration of 2 mg. of 3-methylcholanthrene, in the presence of varying amounts of 17-methyltestosterone, resulted in significant enhancement of androgenic activity of the latter at all dose levels (Fig. 6A). Animals employed in these latter studies were 27 days older than animals used for previous studies and were less sensitive to the action of orally administered 17-methyltestosterone.

As a result of the above investigations, it became of interest to determine if oral 3-methylcholanthrene would induce an irreversible change in cells causing them to become more sensitive to the androgenic effects of 17-methyltestosterone. 3-Methylcholanthrene (2 mg.) was administered orally for 10 days to 55-day old castrate male rats; animals were subsequently allowed to remain untreated for an additional 10 days; 5 mg. of 17-methyltestosterone were thereupon administered for an additional 10 days. Examination of Figure 6B reveals that potentiation of androgenic effects of 17-methyltestosterone did not persist following the 10-day withdrawal period. Thus, the influence of 3-methylcholanthrene on augmentation of biological response to 17-methyltestosterone is not irreversible, but exists only during administration of the carcinogen.

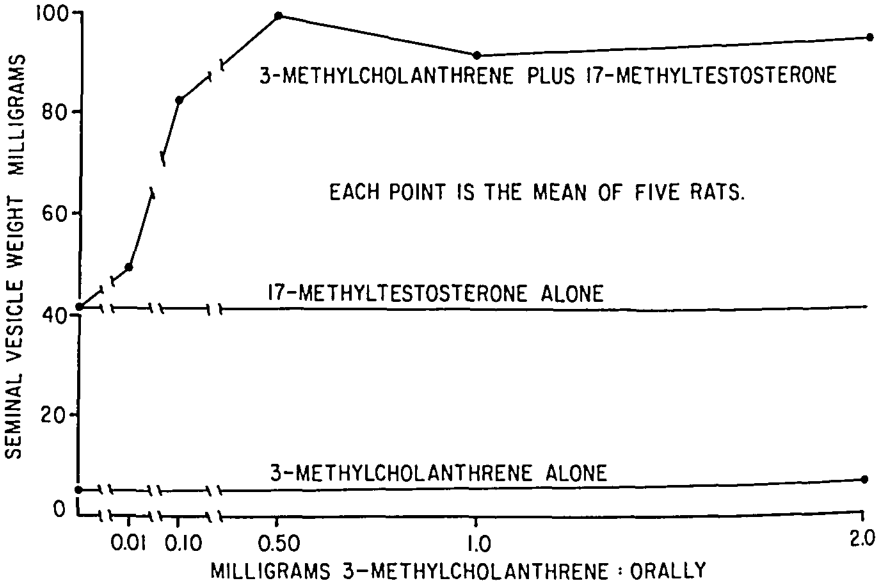

FIG. 6. Effect of orally administered 3-methylcholanthrene on response of seminal vesicle to 17-methyltestosterone. *Conditions:* Male rats (26–28 days old) castrated on day 1. 3-Methylcholanthrene administered orally in varying amounts for 8 days. 17-Methyltestosterone administered orally for 8 days. Animals autopsied on the 9th day.

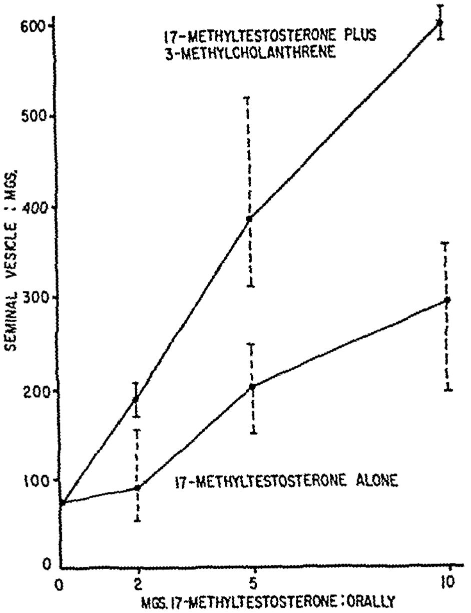

FIG. 6A. Effect of 3-methylcholanthrene administration on seminal vesicle response to 17-methyltestosterone. *Conditions:* 55-Day-old male rats castrated on day 1. Compounds administered orally starting on day 2 and continued for 10 days. Autopsy on day 11. Each point is the mean of 5 rats. Bars represent variation from the mean.

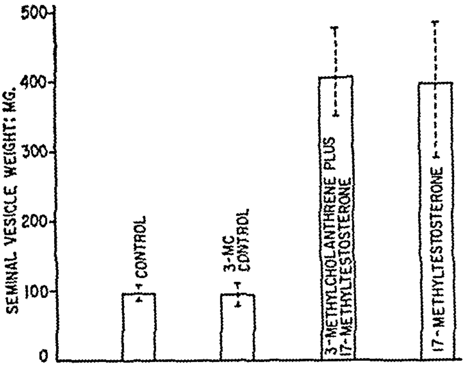

FIG. 6B. Effect of prior administration of 3-methylcholanthrene (3-MC) on response of seminal vesicle to 17-methyltestosterone. *Conditions:* 55-Day-old male rats castrated on day 1. 3-Methylcholanthrene administered on day 2 and continued for 10 days. 17-Methyltestosterone administered 10 days following cessation of 3-methylcholanthrene administration and continued for 10 days. Autopsy 30 days following initial injection with 3-methylcholanthrene. Bars indicate the mean variation: 5 rats per group.

Administration of 3-methylcholanthrene (2 mg./day for 20 days) to intact male rats (20) did not augment the action of endogenously secreted androgenic hormones on the seminal vesicle. Relative growth rates of seminal vesicles for both control-treated and 3-methylcholanthrene-treated animals were identical.

It became of interest to determine if 3-methylcholanthrene administration would result in augmentation of 17-methyltestosterone at dose levels of the latter where it no longer exerts an increased biological effect. Varying amounts of 17-methyltestosterone were administered orally with 3-methylcholanthrene (2 mg.). Examination of Table 2 reveals that, although 3-methylcholanthrene administration induces the expected augmentation of biological response to 17-methyltestosterone, it fails to do so at dose levels of the latter which alone produce a maximal biological effect.

When 3-methylcholanthrene and testosterone propionate were both administered subcutaneously, it was discovered that the hydrocarbon significantly potentiated the "androgenic" effects of this steroid (Fig. 7). However, the amount of augmentation produced by this route of administration was considerably less than following oral administration. These

TABLE 2. EFFECT OF 3-METHYLCHOLANTHRENE ADMINISTRATION ON RESPONSE OF LEVATOR ANI MUSCLES AND SEMINAL VESICLES OF CASTRATE MALE RATS TO 17-METHYLTESTOSTERONE

	Treatment	No. rats	Dose/Day (orally)	Seminal vesicle weight (mg.)	Levator ani weight (mg.)
A	None	5	—	7	30
B	3-Methylcholanthrene	5	20 mg./kg.	10	28
C	17-Methyltestosterone	5	10 mg./kg.	30	42
D	17-Methyltestosterone	5	20 mg./kg.	59	60
E	17-Methyltestosterone	5	40 mg./kg.	78	61
F	17-Methyltestosterone	5	80 mg./kg.	131	77
	B+C	5		58	59
	B+D	5		83	57
	B+E	5		116	72
	B+F	5		130	78

results may be due to inadequate absorption of 3-methylcholanthrene, since nodules containing injected material were found at autopsy. The effects of testosterone propionate on the levator ani were not significantly augmented by simultaneous administration of 3-methylcholanthrene.

Oral administration of 2-anthramine, benz-α-pyrene and 7-methyl-

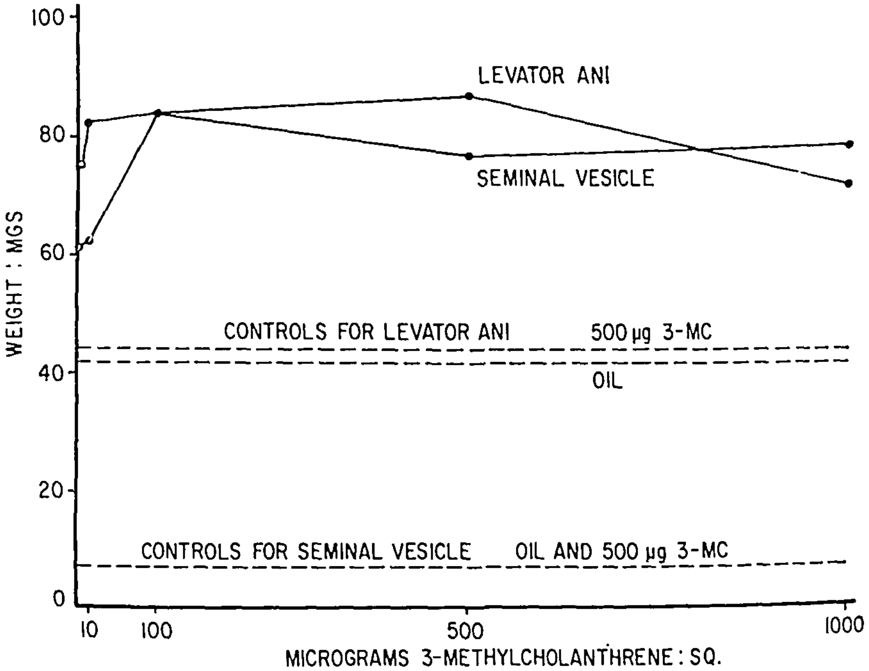

FIG. 7. Effect of 3-methylcholanthrene on the seminal vesicle and levator ani response to subcutaneously administered testosterone propionate. *Conditions:* Castrate rats 26–28 days old. Injections started on day 1 and continued for 20 days. Testosterone propionate 25 μg. per day per rat. Animals autopsied on day 21. Each point is the mean of 6 rats.

benzanthracene, all carcinogenic agents, potentiated the androgenic and myotrophic effects of 17-methyltestosterone, while noncarcinogenic agents, anthracene, phenanthrene, anthrone and anthroquinone, failed to potentiate significantly the biological effects of testosterone propionate or of 17-methyltestosterone (Table 3). Deoxycholic, methyldeoxycholic, and dehydrocholic acids were used as additional controls for the above studies and were also found to be devoid of significant activity (Table 3).

TABLE 3. EFFECT OF BILE ACIDS, CARCINOGENIC HYDROCARBONS, AND OTHER AGENTS ON THE RESPONSE OF THE SEMINAL VESICLE AND LEVATOR ANI TO TESTOSTERONE DERIVATIVES

Compound	Dose	Route	Seminal vesicle (wt., mg.)	Levator ani (wt., mg.)
1. Methyltestosterone	2.0 mg./day	Oral	30.0	57.0
2. 2-Anthramine	0.5 mg./day	Oral	6.0	30.0
Nos. 1 & 2			77.0	75.0
3. Benz-α-pyrene	0.5 mg./day	Oral	7.0	27.0
Nos. 1 & 3			66.0	78.0
4. 7-Methylbenzanthracene	1.0 mg./day	Oral	7.0	32.0
Nos. 1 & 4			79.0	72.0
5. Anthrone	2.0 mg./day	Oral	6.0	30.0
Nos. 1 & 5			39.0	61.0
6. Anthraquinone	5.0 mg./day	Oral	6.0	30.0
Nos. 1 & 6			38.0	49.0
1. Testosterone propionate	25 μg./day	SC	61.0	74.0
2. Anthracene	100 μg./day	SC	7.0	50.0
Nos. 1 & 2			60.0	75.0
3. Phenanthrene	100 μg./day	SC	6.0	56.0
Nos. 1 & 3			54.0	72.0
Cottonseed oil	0.2 ml./day	Oral	7.0	31.0
1. Methyltestosterone	2.0 mg./day	Oral	27.0	49.0
2. Deoxycholic acid	5.0 mg./day	Oral	7.0	33.0
Nos. 1 & 2			38.0	52.0
3. Deoxycholic acid (methyl ester)	5.0 mg./day	Oral	5.0	29.0
Nos. 1 & 3			33.0	44.0
4. Dehydrocholic acid	5.0 mg./day	Oral	5.0	30.0
Nos. 1 & 4			33.0	49.0

Conditions: Six animals per group. Castrate male rats, 26–28 days old. Compounds administered orally for 9 days or subcutaneously for 20 days. On days 10 and 21 animals sacrificed and levator ani muscle and seminal vesicles weighed on precision balance.

DISCUSSION

Studies have been presented to show that oral administration of the carcinogenic hydrocarbons, 3-methylcholanthrene, 2-anthramine, benz-α-pyrene, and 7-methylbenzanthracene, results in marked potentiation of the androgenic or myotrophic effects of 17-methyltestosterone. 3-Methylcholanthrene also potentiates the biological action of hydrocortisone on two separate end points—liver glycogen and granuloma pouch. Observations that four unrelated organs, the levator ani muscle, seminal vesicle, liver and granuloma pouch, respond in similar fashion to administered carcinogenic agents indicate a type of cellular activity which may be unrelated to the type of organ response ordinarily associated with specific activities. That is, carcinogenic hydrocarbons may elicit fundamental cellular changes in all organs and tissues. It appears logical to suppose that carcinogenic hydrocarbons are involved in a generalized cellular alteration, permitting cells to become more sensitive to naturally-occurring agents. Such ideas are merely speculative, but it may be postulated that tumorigenic hydrocarbons are not cancer-inducing *per se*, but merely alter cells in a manner which depresses normal control mechanisms, thus allowing cells to lose their regulatory capacity toward specific agents.

The increased sensitivity of cells to naturally-occurring agents, following oral administration of carcinogenic compounds, may be reflected by systems other than those responsive to steroid hormones. The above experiments suggest a technological mode of attack on the problem of determining the influence of various etiological factors concerned with the tumor process. Knowledge of a useful fundamental character should result from studies designed to determine the types and incidence of tumors following oral administration of carcinogenic hydrocarbons and various agents such as viruses and hormones.

The results reported here are difficult to reconcile with those described by Jackson and Robson (1) who applied 3-methylcholanthrene directly to target organs and reported that this agent *inhibited* the response of specific end organs to both estrogens and androgens. The only control implantation was that which employed cholesterol; not, as in our study, inactive, but related, hydrocarbons. It is important in situations of this type to define the specificity of the material being investigated. Direct addition of any substance to a tissue may produce results not seen when the substance reaches the tissue by normal channels—especially if the substance is as insoluble as are carcinogenic hydrocarbons. Inhibition of a response under such conditions may be the result of nonspecific physical factors and not a reflection of specific biological effects. On the other hand, our own results with carcinogenic agents could be attributed to a bioconversion of carcinogenic hydrocarbons in the liver or other tissues to agents which themselves are responsible for the potentiation of hydrocortisone and 17-methyltestosterone.

On the basis of the existing data, it may be postulated that oral administration of carcinogenic hydrocarbons "sensitizes" cells to the action of a number of steroids at different levels of tissue organization. It may also be postulated that oral administration of carcinogenic agents may "sensitize" cells to a wide variety of agents. For example, Castellano and D'Amour (12) found that administration of stilbestrol increased the incidence of uterine tumors following implantation of 3-methylcholanthrene. Under certain conditions, the estrogens themselves are carcinogenic (13). If 3-methylcholanthrene potentiates the biological action of estrogens, these latter results are readily interpreted on the basis of observations presented in this communication.

That carcinogenic hydrocarbons may "sensitize" cells to the action of other agents is readily apparent from work reported by Berenblum (14). This investigator showed that croton oil applied alone to the skin of mice induces hyperplasia, but normally no tumors develop during the animals' lifetime. If dimethylbenzanthracene is applied alone, hyperplasia develops and, after a prolonged period, some tumors develop. If both croton oil and dimethylbenzanthracene are applied together, tumors appear more rapidly and with greater frequency than when dimethylbenzanthracene alone is applied. Even more striking is the fact that if just one application of dimethylbenzanthracene is made, tumors appear after a protracted period. But if, after the application of dimethylbenzanthracene, croton oil is applied, tumors develop more rapidly, and there is a strong tendency for tumors to appear at a standard time following croton oil application rather than following application of dimethylbenzanthracene. It appears evident that dimethylbenzanthracene causes some irreversible change in cells which remains latent until the application of croton oil. It is interesting in this regard that croton oil-induced inflammation, used in our experiments, and which is inhibited by hydrocortisone, is inhibited even more significantly in the presence of 3-methylcholanthrene. These appear to be actions of opposite extreme attributable to a "conditioning" response brought about by a carcinogenic hydrocarbon. It may well be that carcinogenic agents will produce effects which are either inhibitory or stimulatory depending upon additional circumstances imposed during their administration. As a matter of interest, as well as some practical importance, it may be visualized that the action of carcinogenic hydrocarbons is involved with a type of cellular alteration which exists for prolonged periods following their administration. This type of alteration may never become apparent until the necessary stimulus for cellular proliferation, virus, hormone or foreign agent, is permitted contact with the altered cell. This type of phenomenon may explain the appearance of tumors months or even years after initial contact with carcinogenic agents.

As a matter of theoretical and practical importance it becomes necessary to determine if chemical or physiological means exist which, when applied, will prevent some of the cellular "conditioning" responses to carcinogenic agents such as those illustrated herein. Such information, if it exists experimentally, should be carried over to prophylactic treatment of experimental animals known to have a high incidence of spontaneous tumors. Such efforts will determine if the incidence of tumors can be decreased or completely abolished. Another avenue of approach is one which attempts to more clearly define some of the etiological factors in tumor formation. Combined administration of carcinogenic compounds and naturally-occurring agents such as viruses and hormones should result in development of tumors at a faster than normal rate as well as the appearance of newer types of tumors.

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A METHOD FOR THE EVALUATION OF ADRENOCORTOCOTROPIC HORMONE SUPPRESSING ACTION OF CORTICOIDS[1]

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ABSTRACT

A simple, reproducible method employing the rat is described which is suitable for the evaluation of compounds as to their adrenocorticotropic hormone suppressing action. The endpoint is the adrenal corticosterone content which reflects the concentration of circulating ACTH. With excessive doses of cortisone acetate a 93% decrease in adrenal corticosterone was found. The relative potency of a compound could be determined with a standard error of ±18% by using 20 rats for the standard and 20 rats for the unknown.

IT IS well known that the administration of various corticosteroids causes a decreased elaboration of adrenocorticotropin (ACTH) from the pituitary of various animals. This, in turn, leads to a decreased biosynthesis and release of adrenocorticoids as is reflected also in the hypotrophic and subfunctional state of the adrenal gland. Holzbauer (1) recently presented good evidence showing that the corticosterone stores of the rat adrenal gland reflect the secretion rate of corticosteroid. This in turn reflects the level of circulating ACTH since high or low corticosterone values are obtained when conditions leading to large or small amounts of circulating ACTH respectively are brought about. Thus, any condition which would interfere with the elaboration of ACTH would also be expected to be reflected in the amount of corticosterone in the rat adrenal gland. Silber and Busch (2) have found that the adrenals of rats 2 weeks after hypophysectomy contained 0.04 μg. corticosterone per adrenal as compared to values of 0.30–0.50 μg. for normal adrenals. The same values as in the adrenals of hypophysectomized animals were found after treatment with cortisone acetate at a level of 6 mg./day subcutaneously for 3 weeks (2). Whereas Sweat (3) used ethanolic solutions of steroids for producing fluorescence in sulfuric acid and Peterson (4) later modified this method, the method used in the present paper (2) consists of extracting the tissue steroid directly into methylene dichloride or chloroform. The extracted steroid is then allowed to react with sulfuric acid by adding the latter directly to the methylene dichloride. This communication demon-

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strates that this simple fluorometric method for the direct determination of adrenal corticosterone can be used as the basis of a satisfactory bioassay for the evaluation of the ACTH suppressing action of corticoids.

MATERIALS AND METHODS

Animals and protocols

The steroids suspended in Special Formula No. 17874[2] were injected subcutaneously into male albino rats weighing 100–175 gm. The daily dose was contained in 0.25 ml. and control rats received only the vehicle. On the day following the last injection each rat was treated with nembutal, taking every precaution to handle the animals as gently as possible (1) and the adrenals removed, cleaned of adherent fat, and weighed.

Fluorometric method

The method of extracting the steroid from the tissue and development of the fluorescence in the extracts of tissue is given elsewhere (2). This method has formed the basis also for the measurement of corticosterone in rat plasma by Guillemin *et al.* (5). The corticosterone content of the rat adrenals was determined by measuring the fluorescence emitted with the Aminco-Bowman Spectrophotofluorometer. The activation monochromator was set at λ max 470 mμ and the fluorescence monochromator at λ max 525 mμ. These are the uncorrected fluorometric maxima. Samples were read on the photomultiplier microphotometer with the sensitivity knob set at the maximum position and the meter multiplier at the 1.0 position. The slit arrangement was No. 5. The fluorescence was proportional to the amount of corticosterone used (0.02–0.08 μg.) for the standard curve and the photomultiplier reading was in the neighborhood of 35 for the 0.08 μg. concentration with the settings as stated. A standard curve was run with each experiment. Figure 1 shows the data of recovery experiments plotted graphically wherein several concentrations of corticosterone were added to a fixed weight of adrenal tissue (10 mg.). The reliability of the method was tested further by running converse experiments wherein the amount of adrenal tissue was varied but the amount of corticosterone added to it kept constant. Figure 2 shows that additions of 0.04 μg. corticosterone (Experiments 1 and 2) and 0.08 μg. (Experiments 3 and 4) to varying amounts of adrenal tissue (1.25 ml. of tissue aliquot = 10 mg. of adrenal tissue) does not interfere with the extraction of corticosterone or its recovery from the tissue. Since the experimental curves are linear and their slopes proportional to the amount of tissue used, after subtracting the value found for the added corticosterone, this indicates that the quenching phenomena often observed in fluorometric determinations are absent. Table 1 gives data showing the specificity of the method by indicating the very low apparent concentrations of corticosterone-like compound found in extra adrenal tissue.

RESULTS AND DISCUSSION

Table 2 shows the concentration of corticosterone[3] found in the adrenals of control rats and those treated for a 21-day period with 9α-fluorocorti-

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[2] Special Formula No. 17874 is a product obtained from the Upjohn Co., Kalamazoo, Michigan. It is a mixture of carboxymethycellulose, polysorbate 80, sodium chloride and benzyl alcohol in water for injection.

[3] It is assumed that the steroid giving rise to fluorescence is corticosterone since very small amounts of other steroids are found in the rat adrenal (1). This also excludes aldosterone which is not detected by procedure (6) although this steroid is released in a ratio of about 10/1 of corticosterone to aldosterone in the incubation medium of rat adrenals incubated in Krebs-Ringer bicarbonate-glucose buffer (7).

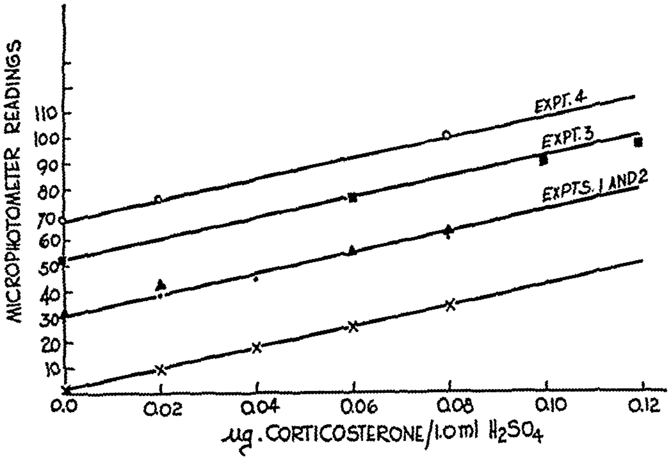

FIG. 1. Recovery Experiment. X——X Standard curve for corticosterone 0.02–0.08 μg. Other curves represent those obtained when various amounts of corticosterone (0.02–0.12 μg.) are added to 10 mg. of rat adrenal tissue. The difference between the blank value of the standard curve and the values for the experiments on the ordinate are equal to the readings given by the tissue alone. Markings on the Experiment 1–4 curves (▲, ●, ■, ○) show the additions of corticosterone at the concentration denoted on the abscissa. Parallelism of all curves with that of the standard curve denotes good recovery.

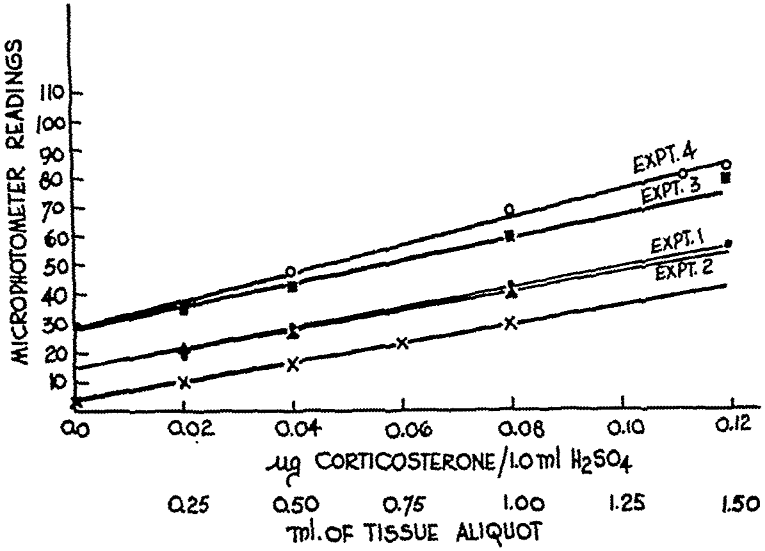

FIG. 2. Modified Recovery Experiment. X——X Standard curve for corticosterone 0.02–0.08 μg. The double plot on the abscissa denotes the concentration of corticosterone used for the standard curve (μg. corticosterone/1.0 ml. H_2SO_4) and the amount of tissue used in Experiments 1–4 (ml. of tissue aliquot). Markings on the Experiment 1–4 curves (▲, ●, ■, ○) show the amount of tissue aliquot used in each experiment. 0.04 μg. of corticosterone (Experiments 1 and 2) and 0.08 μg. of corticosterone (Experiments 3 and 4) were added to the aliquots of tissue used for analysis. Extrapolation points of the curves of Experiments 1 and 2 on the ordinate when read on the standard curve should read 0.04 μg. corticosterone and by the same token 0.08 μg. for Experiments 3 and 4.

TABLE 1. APPARENT CORTICOSTERONE CONCENTRATION IN DIFFERENT TISSUES OF THE RAT

Tissue	Wet weight, mg.	Total corticosterone, μg.
Liver	9.6	0.010
	10.4	0.010
	20.3	0.014
	20.0	0.016
	40.0	0.016
	41.0	0.016
Kidney	11.4	0.010
	10.0	0.010
	20.6	0.016
	22.0	0.015
	41.0	0.015
	42.0	0.019
Brain	11.4	0.017
	11.2	0.016
	20.8	0.021
	21.2	0.021
	41.0	0.029
	41.0	0.029

sone acetate and cortisone acetate respectively. Nine of the ten rats injected with 9α-fluorocortisone acetate did not survive the injection period while two died in the group receiving the cortisone acetate. The over-all results indicate clearly the greatly decreased content of corticosterone in the rat adrenals as a result of steroid administration. Cortisone acetate caused a 93% decrease in adrenal corticosterone and a 60% decrease in adrenal weight reduction and essentially confirms the findings of Silber and Busch (2). In the single survivor of 9α-fluorocortisone acetate treatment a similarly low adrenal corticosterone was found. Analysis of weight and adrenal corticosterone content of the left and right glands revealed no significant difference (Table 2).

Table 3 shows the results obtained when cortisol was injected over a fairly wide range of concentrations in seven trials. The minimum dosage level at which cortisol caused a significant decrease in adrenal cortico-

TABLE 2. INFLUENCE OF CORTISONE ACETATE AND 9α-FLUOROCORTISONE ON THE CORTICOSTERONE CONTENT OF RAT ADRENALS

(Treatment period was 21 days)

Material administered	Total dose, mg.	No. of rats	Mean adrenal weight, mg. ±S.E.		Mean corticosterone in gland, μg. ±S.E.	
			Left adrenal	Right adrenal	Left adrenal	Right adrenal
0	0	10	18.1±1.0	19.9±0.9	1.33±0.13	1.31±0.14
Cortisone Acetate	48.3	18*	8.6±1.0	8.7±0.7	0.09±0.008	0.10±0.120
9α-Fluorocortisone Acetate	21	1†	8.5	8.0	0.09	0.09

* 2 animals died during experimental period.
† 9 animals died during experimental period.

TABLE 3. INFLUENCE OF CORTISOL ON THE CORTICOSTERONE CONTENT OF RAT ADRENALS
(Treatment period was 7 days)

Material administered	Total dose, mg.	No. of rats	Mean corticosterone per adrenal, μg. ±S.E.
0	0	12	0.68 ±0.03
Cortisol	0.70	12	0.77 ±0.09
	2.10	11	0.72 ±0.06
	6.30	11	0.45 ±0.03
0	0	12	0.80 ±0.06
Cortisol	0.70	12	0.88 ±0.10
	2.10	12	0.78 ±0.08
	6.30	11	0.41 ±0.04
0	0	12	0.76 ±0.07
Cortisol	6.30	9	0.48 ±0.06
	12.60	10	0.26 ±0.02
	18.90	11	0.26 ±0.01
0	0	12	0.92 ±0.08
Cortisol	6.30	12	0.52 ±0.05
	12.60	12	0.29 ±0.02
	18.90	12	0.27 ±0.03
0	0	6	0.83 ±0.19
Cortisol	3.15	12	0.71 ±0.07
	6.30	12	0.55 ±0.07
	12.60	12	0.34 ±0.01
0	0	11	0.82 ±0.05
Cortisol	3.15	12	0.68 ±0.04
	6.30	12	0.38 ±0.03
	12.60	12	0.27 ±0.01
0	0	12	1.13 ±0.07
Cortisol	3.15	12	0.82 ±0.05
	6.30	12	0.74 ±0.07
	12.60	12	0.36 ±0.02

sterone was found to be 3.15 mg. per 7 days, while 12.60 mg. per 7 days caused a maximum decrease. The decrease in adrenal corticosterone was proportional to the amount of cortisol administered between the limits of 3.15 to 12.6 mg. per animal over the 7-day period. In six trials slopes rang-

TABLE 4. INDEX OF PRECISION OF ASSAY USING CORTISOL

N	Total dose levels, mg.	Slope b	Index of precision λ
22	2.1; 6.3	−0.572	0.260
23	2.1; 6.3	−0.711	0.296
19	6.3; 12.6	−0.758	0.158
24	6.3; 12.6	−0.773	0.149
36	3.15; 6.3; 12.6	−0.615	0.276
36	3.15; 6.3; 12.6	−0.764	0.222

ing from −0.572 to −0.773 were found with a mean of −0.699. The index of precision (λ) varied from 0.149 to 0.296 for these trials with a mean of 0.227 (Table 4). On the basis of these calculations it appears that the relative potency of a compound could be determined in relation to cortisol with a standard error of ±18% by using a total of 40 animals, 20 on the unknown and 20 on the standard.

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ADRENAL CORTICOID ACTIVITIES OF 9α-FLUORO-11β,16α,17α,21-TETRAHYDROXY-1,4-PREGNADIENE-3,20-DIONE[1,2,3]

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ABSTRACT

Triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) was studied in rats to assess its adrenocortical activities using the following assays: electrolyte excretion, liver glycogen deposition, growth-survival and anti-inflammatory activity (asbestos pellet technique).

Subcutaneously administered triamcinolone was 3 to 12 times more potent than prednisolone and 10 to 40 times more active than hydrocortisone in liver glycogen deposition tests. On oral assay, the relative potency of triamcinolone was higher.

Triamcinolone proved to be 5 to 10 times more active than hydrocortisone acetate in the anti-inflammatory assay.

Triamcinolone was more efficacious than hydrocortisone and at least as effective as DOC in maintaining the life of immature, male, adrenalectomized rats. Although survival time was prolonged, there was a suppression of growth when compared with the DOC-treated rats.

Triamcinolone was found to be a potent diuretic and natriuretic steroid.

EFFORTS to synthesize useful therapeutic analogs of hydrocortisone and cortisone have resulted in numerous steroids possessing pronounced mineralo- and glucocorticoid activities. Structural modifications of hydrocortisone which enhanced glucocorticoid activity include halogenation at carbon 9 (1, 2), introduction of a double bond between carbon 1 and 2 (3–6), and methylation at either carbon 2 (7, 8) or carbon 6 (9).

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[1] These experiments were conducted in the Pharmacological Research Department (Dr. Earl H. Dearborn, Department Head) of the Experimental Therapeutics Research Section (Dr. John T. Litchfield, Jr., Director) Research Division, American Cyanamid Company, Pearl River, New York.

[2] This work was initiated through the efforts of Drs. Fred I. Dessau and Paul H. Bell. The authors wish to express their gratitude to these investigators for their aid and advice in pursuance of this study. The authors are also indebted to Dr. Seymour Bernstein and associates for the generous supplies of triamcinolone and to Messrs. C. W. Dunnett and J. D. Haynes for the statistical evaluation.

[3] A preliminary report of this work was presented before the Society of Pharmacology and Experimental Therapeutics, Baltimore, Md., 1957. *J. Pharmacol. and Exper. Therap.* **122,** 60A, 1958.

Further augmentation of activity was accomplished by combinations of two or more of the above molecular modifications (8, 10, 11); however, an increase in electrolyte activity (sodium retention-potassium excretion) is associated with innovations such as 9α-halogenation and 2α-methylation of hydrocortisone. Prednisolone (Δ^1-hydrocortisone) reported to be three times as potent as hydrocortisone in animal assays, "failed to produce sodium retention in adrenalectomized rats" (6), whereas 6α methyl-Δ^1-hydrocortisone, also a potent glucocorticoid, was diuretic and natriuretic (10).

In the quest for further interesting chemical substitutions, investigations in the biosynthesis of adrenal corticoids by a variety of species have suggested the presence of compounds more polar than hydrocortisone (12). Considerable interest has been shown in corticoids possessing additional hydroxyl groups at positions 6 and/or 16 (13, 14). In a review article Bernstein (15), presents a list of some fourteen steroids, hydroxylated in the 16 position, which have been isolated from urine and characterized.

16α-Hydroxy desoxycorticosterone was first prepared in 1953 by Hirschmann, Hirschmann, and Farrell (16), who observed that the "compound failed to demonstrate the high salt retaining activity of electrocortin." Others, who have also reported on this compound, found that sodium-retention activity is absent or weak (17, 18).

In further pursuance of this modification as a means of dissociating the undesirable sodium retentive activity of certain potent glucocorticoids, a number of 16α-hydroxylated compounds have been prepared by Bernstein and his collaborators (19–23). The most active, 9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione (triamcinolone) possessed a glucocorticoid potency 3 to 12 times that of prednisolone. Furthermore, triamcinolone exhibited a marked ability to promote water and sodium diureses.

An elucidation of the biological efficacy of this compound in comparison with other steroids is the aim of the experiments reported.

METHODS

Urinary excretion and liver glycogen deposition assay

In this assay, the biological effects of adrenocorticoids on electrolyte excretion and liver glycogen deposition were assayed simultaneously in the same animal.

Steroid suspensions were prepared by grinding the steroids with the vehicle in ground glass homogenizers. The vehicle contained 0.5% carboxymethylcellulose and 0.4% Tween 80 in 0.9% sodium chloride solution. This modification of the medium of Stafford *et al.* (11) excludes benzyl alcohol.

Adult male rats (Sherman or Wistar strain, 140–170 Gm.) were bilaterally adrenalectomized, and were given 1% sodium chloride in their drinking water and the high protein diet recommended by Olson *et al.* (24). All animals were held for 4 days after adrenalectomy to allow clearance of residual adrenal steroids. On the fourth postoperative day, the animals were weighed and randomly grouped, and the food was removed to insure maximum depletion of liver glycogen. Twenty-four hours later, the saline was discontinued, and graded doses of the steroids, in 0.2 ml. of vehicle per dose, were injected subcutaneously, or in 0.5 ml. of vehicle per dose, were administered orally by gavage. Control rats received only the carboxymethylcellulose vehicle.

Thirty minutes after the administration of the steroid, the rats were lightly etherized to insure complete emptying of the bladder. They were then injected intraperitoneally with 5 ml. of 0.9% sodium chloride solution, and two rats were placed in each metabolism cage. The urine was collected in graduated cylinders after passing through a glass wool filter placed in the mouth of the funnel to prevent fecal contamination. No food or water was allowed during the assay. Five hours after saline administration, the glass wool filters were squeezed to remove trapped urine and the volume was recorded. The cages and funnels were then rinsed with distilled water until the final volume was 25 ml. The milliequivalents of urinary sodium and potassium per pair of rats were determined by flame photometry.

Seven hours after dosing, all animals were anesthetized with 40 mg. of sodium pentobarbital per kilogram of body weight. The livers were quickly excised and immediately hydrolyzed in 30% potassium hydroxide (Pabst *et al.* (25)). The total liver glycogen was determined by the anthrone method of Seifter *et al.* (26).

The relative potencies of the steroids involved were statistically analyzed by determining the linear regression lines by the method of least squares. Since these lines were not parallel, the relative potencies varied with the response level. The 0.4 and 1.2% liver glycogen points were arbitrarily chosen as the response levels at which the potencies are quoted.

Life maintenance and growth

Immature male rats (40–60 gm.) were bilaterally adrenalectomized and immediately injected subcutaneously with graded doses of the steroids suspended in 0.2 ml. of carboxymethylcellulose vehicle. Both sham-operated (rats whose adrenals were exposed but not removed, under identical operating conditions) and adrenalectomized controls received vehicle only. The injections continued daily for 14 days. The animals were maintained on Purina dog chow and tap water *ad libitum.*

Weights were recorded every 3 days throughout the 28-day period of the assay. Animals were checked daily and any rats surviving to the 28th day, with the exception of those sham-operated, were autopsied and examined for residual adrenal tissue. Those found to have evidence of adrenal tissue were eliminated from the experiment. This experimental procedure was modified from that described by Tolksdorf *et al.* (6).

Anti-inflammatory test

The test system is a modification of Meier's cotton pellet method (27). Female albino rats (100–120 gm.) were anesthetized, and a 30 mg. ± 1 mg. asbestos pellet (Seitz S-1, sterilizing) was placed in each of two subcutaneous pockets in the left and right dorsal areas. Twice daily, beginning within two hours postoperatively, groups of 6 rats were dosed subcutaneously with 0.1 ml. of the steroid suspension. The concentrations of the steroid suspensions were such that one half the desired daily dose was contained in 0.1 ml. The control group, consisting of twice as many animals as the test group, was treated twice daily with 0.1 ml. of vehicle. Treatment was continued for 5 days. On the 6th day, the rats were sacrificed and the pellets, with accompanying granuloma tissue, were excised and weighed.

Statistical analyses similar to those employed in the liver glycogen studies were used to determine the relative potencies. Here again, the regression lines were not parallel so the 600 and 1000 mg. pellet weights were chosen as the response levels for comparison.

RESULTS

Electrolyte studies

In contrast to desoxycorticosterone, triamcinolone exhibited a marked ability to promote sodium excretion when administered either subcutaneously or orally to adrenalectomizde rats (Tables 1 and 2). Rats receiving 50 μg. orally more than doubled their excretion of urine and increased urinary sodium excretion by 62% as compared with adrenalectomized controls. At the same time, potassium loss was increased by 59%. Further evaluation of the data indicated that, generally, water and potassium excretion increased over control levels to a greater degree than that of sodium.

TABLE 1 5-HOUR URINARY EXCRETION FOLLOWING SUBCUTANEOUS ADMINISTRATION OF STEROIDS TO FASTED, SALINE-LOADED, ADRENALECTOMIZED RATS*

Compound	Dose, mg	No † rats	Urine volume		Electrolyte response			
					Na^+		K^+	
			ml /5 hrs (S E)‡	% of cont	m eq /5 hrs (S E)	% of cont	m eq /5 hrs (S E)‡	% of cont
Control	—	10	5 1 (±1 4)	100	1 55 (± 4)	100	0 20 (± 01)	100
DOC	.05	8	5 6 (± 37)	109	1 09 (± 12)	70	0 34 (± 02)	170
	.10	4	6 7 (6 0–7 5)§	131	1 25 (1 08–1 42)§	80	0 34 (34– 34)§	170
Triamcinolone	006	8	8 6 (±1 3)	168	1 74 (± 18)	112	0 30 (± 05)	150
	025	8	15 4 (±1 3)	302	2 27 (± 18)	146	0 44 (± 03)	220
	05	8	10 6 (±1 1)	208	1 93 (± 30)	124	0 55 (± 06)	275
	10	8	11 7 (±0 77)	229	2 19 (± .15)	141	0 65 (± 03)	325

* Pooled data from duplicate experiments
† 2 rats/cage—values expressed/cage
‡ Standard Error
§ Range

TABLE 2 5-HOUR URINARY EXCRETION FOLLOWING ORAL ADMINISTRATION OF STEROIDS TO FASTED, SALINE-LOADED, ADRENALECTOMIZED RATS*

Compound	Dose mg	No † rats	Urine volume		Electrolyte response			
					Na^+		K^+	
			ml /5 hrs. (range)	% of cont	m eq /5 hrs (range)	% of cont	m eq /5 hrs (range)	% of cont
Control	—	8	3 5 (2 5– 5 5)	100	0 96 (75–1 26)	100	0 22 (19– 26)	100
DOC	05	4	4 0 (1 5– 6 5)	115	1 14 (71–1 57)	118	0 30 (29– 31)	137
	25	4	3 0 (1 5– 4 5)	86	1 09 (81–1 38)	113	0 23 (22– 24)	105
	1	4	2 7 (2 5– 3 0)	80	0 89 (87– 92)	92	0 21 (20– 21)	95
	5	4	2 7 (2 5– 3 0)	80	0 82 (74– 91)	85	0 43 (37– 49)	195
Triamcinolone	01	4	7 5 (6 0– 9 0)	215	1 36 (1 22–1 50)	142	0 21 (20– 21)	95
	05	8	7 9 (3 0–11 5)	226	1 55 (88–2 09)	162	0 35 (34– 39)	159
	25	8	8 1 (7 0– 9 0)	231	1 57 (1 23–1 94)	163	0 51 (46– 59)	232
	1 25	4	8 0 (5 0–11 0)	228	1 93 (1 84–2 02)	202	0 67 (65– 69)	304

* Pooled data from duplicate experiments
† 2 rats/cage—values expressed/cage.

Comparable data on the diuretic and natriuretic actions of triamcinolone have been obtained by Dorfman *et al.* (28).

Liver glycogen deposition studies

Triamcinolone and prednisolone were compared with hydrocortisone for liver glycogen deposition activity after subcutaneous or oral administration (Tables 3 and 4). Triamcinolone was found to be 10 (95% fiducial limits 7 to 15) to 40 (23 to 69) times as potent as hydrocortisone subcutaneously, and 41 (30 to 56) to 84 (60 to 117) times hydrocortisone orally. The relative potency of triamcinolone to prednisolone in the subcutaneous and oral assay was calculated to be 3 (2 to 5) to 12 (8 to 20), and 9 (7 to 13) to 19 (14 to 27), respectively. Triamcinolone diacetate was found to be 9

TABLE 3 LIVER GLYCOGEN DEPOSITION FOLLOWING SUBCUTANEOUS ADMINISTRATION OF STEROIDS TO FASTED, ADRENALECTOMIZED RATS—7-HOUR ASSAY*

Compound	Dose (mg /rat)	No of rats	Mean liver glycogen %	Standard error of mean
Control	—	14	0 25	± 02
Hydrocortisone	25	4	0 52	± 07
	50	4	1 04	± 04
	1 00	12	1 39	± 12
Prednisolone†	125	8	0 83	± 10
	25	6	1 55	± 27
	50	8	1 54	± 01
	1 00	7	1 64	± 06
Triamcinolone	006	8	0 36	± 08
	025	7	0 79	± 09
	050	8	1 15	± 06
	100	8	1 32	± 07
Triamcinolone diacetate	006	7	0 31	± 04
	025	8	0 95	± 04
	050	8	1 06	± 08
	100	8	1 19	± 03

* Pooled data from duplicate experiments
† Obtained from Schering Corp , Bloomfield, N J

TABLE 4 LIVER GLYCOGEN DEPOSITION FOLLOWING ORAL ADMINISTRATION OF STEROIDS TO FASTED, ADRENALECTOMIZED RATS—7-HOUR ASSAY*

Compound	Dose (mg /rat)	No of rats	Mean liver glycogen %	Standard error of mean
Control	—	8	0 22	± 01
Hydrocortisone	2	4	0 99	± 23
	6	8	1 33	± 18
	12	8	1 55	± 10
Prednisolone†	0 5	8	0 79	± 03
	2	8	1 52	± 16
	8	8	2 19	± 18
Triamcinolone	0 01	4	0 43	± 06
	0 05	8	1 05	± 10
	0 25	8	1 35	± 14
	1 25	4	1 37	± 15
Triamcinolone diacetate	0 01	4	0 34	± 03
	0 05	8	0 82	± 08
	0 25	8	1 51	± 17
	1 25	4	1 34	± 23

* Pooled data from duplicate experiments
† Obtained from Schering Corp , Bloomfield, N J

(6 to 14) to 37 (22 to 63) times as potent as hydrocortisone subcutaneously, and 36 (26 to 49) to 73 (53 to 102) times hydrocortisone orally.

Life maintenance and growth studies

The data presented in Table 5 (Experiments 1 and 2) are the results of duplicate studies. Adrenalectomized rats had a mean survival time of 5 days. In groups receiving desoxycorticosterone, mean survival times of 7 to 16 days were recorded. Only the sham-operated animals survived the 28-day test. It is evident that hydrocortisone was less effective than desoxycorticosterone in maintaining life, while triamcinolone, triamcinolone diacetate, and prednisolone were at least as effective, if not more so. The results with prednisolone are in accord with those reported by other workers (6).

Changes in body weight observed during steroid treatment are included

TABLE 5. SURVIVAL TIME OF ADRENALECTOMIZED, IMMATURE MALE RATS

Treatment	Dose, mg./rat /day subcut.	No. of rats	Average body weight in gm.		No. surviving 14 days	Mean survival time (days) ±S.E.
			Initial (range)	At 13 days (range)		
			Experiment 1*			
Sham-adrenalectomized controls	—	15	60 (45–70)	130 (114–154)	13	25 ±2.0
Adrenalectomized controls	—	15	60 (50–70)	—	0	5 ± .6
Desoxycorticosterone	0.045	15	59 (48–73)	—	0	7 ± .8
	0.18	16	56 (46–60)	104 (84–118)	11	16 ±1.7
Hydrocortisone	0.12	16	52 (50–68)	96 (96)	1	10 ± .7
	0.48	15	60 (52–70)	93 (70–118)	12	17 ±1.4
Prednisolone†	0.03	16	60 (47–72)	—	0	9 ± .7
	0.12	18	59 (50–71)	80 (58–100)	14	15 ±1.2
Triamcinolone	0.015	15	59 51–64	—	0	8 ± .8
	0.03	15	58 (50–66)	70 (62–78)	3	9 ±1.0
	0.06	17	58 (46–67)	69 (50–85)	12	13 ±1.4

* Wistar strain rats.

† Obtained from Schering Corp., Bloomfield, N. J.

(*Continued on next page*)

TABLE 5. (*Continued*)

Treatment	Dose, mg./rat/day subcut.	No. of rats	Average body weight in gm.		No. surviving 14 days	Mean survival time (days) ±S.E.
			Initial (range)	At 13 days (range)		
	0.12	14	59 (52–69)	68 (60–74)	5	9±1.7
Triamcinolone diacetate	0.015	16	57 (49–67)	56 (56–56)	1	8±0.7
	0.03	16	58 (48–67)	65 (60–69)	3	8±1.2
	0.06	17	58 (52–66)	68 (64–76)	7	11±1.6
	0.12	15	58 (46–65)	64 (54–78)	7	12±2.3
Experiment 2*						
Sham-adrenalectomized controls	—	33	65 (52–78)	131 (103–170)	33	28
Adrenalectomized controls	—	35	62 (52–74)	94 —	1	5± .4
Desoxycorticosterone	0.045	35	65 (54–74)	93 (92–94)	2	7± .5
	0.09	33	62 (50–74)	91 (83–98)	8	9± .7
	0.18	34	62 (44–74)	102 (73–126)	21	14±1.2
Hydrocortisone	0.12	34	62 (52–70)	88 (79–94)	4	9± .6
	0.24	34	64 (53–74)	88 (70–130)	26	15± .8
	0.48	34	63 (50–74)	94 (78–110)	25	15±1.2
Prednisolone†	0.03	36	63 (54–70)	90 (74–105)	6	9± .8
	0.06	33	60 (55–72)	89 (74–112)	8	10± .8
	0.12	36	62 (50–73)	83 (56–108)	26	14±1.0
Triamcinolone diacetate	0.015	34	63 (54–74)	79 (68–93)	6	9± .7
	0.03	32	59 (54–74)	78 (70–88)	13	12± .8
	0.06	34	63 (48–72)	70 (54–94)	22	14±1.0
	0.12	32	63 (52–72)	70 (48–86)	24	17±1.1

in Table 5. Desoxycorticosterone was superior to triamcinolone, triamcinolone diacetate, or prednisolone in promoting the growth of adrenalectomized rats. Moreover, prednisolone appeared superior to triamcinolone or its diacetate in this respect. That a direct relationship between survival and weight gain in adrenalectomized animals need not exist has been expressed by both Dorfman (29) and Leathem (30).

Anti-inflammatory studies

The ability of triamcinolone to inhibit the formation of granulomatous tissue appeared enhanced in comparison with hydrocortisone. It can be seen from the data (Table 6) that there was a decrease in granuloma formation as the dosage of steroid was increased. There was also a concomitant decrease in body weight.

TABLE 6. EFFECT OF STEROID TREATMENT ON GRANULOMA FORMATION

No. animals per group	Chemical	Dose, mg./day	Avg. body weight change, gm.	Avg. granuloma wt. per rat, mg.
12	Control	—	+10.8	1,202
6	Hydrocortisone acetate	12	−13.3	596
6		4	−11.6	682
6		1.4	− 6.6	832
6		0.4	− 3.3	1,064
6		0.14	+10.0	1,166
6	Triamcinolone	1.2	−28.3	552
6		0.4	−15.0	754
6		0.14	− 1.6	916
6		0.04	+ 3.3	1,152

While a decrease in both granuloma formation and body weight occurred during hydrocortisone and triamcinolone treatment, the two phenomena are not necessarily dependent upon each other. Desaulles *et al.* (31) have shown that, while cortisone and hydrocortisone result in a reduction of both granuloma formation and body weights, corticosterone and desoxycorticosterone reduced the granuloma formation while maintaining body weight gains.

The relative potency of triamcinolone was 5 (95 per cent fiducial limits 3 to 8) to 10 (7 to 15) times that of hydrocortisone.

DISCUSSION

Glucocorticoid potency exceeding that of cortisone and hydrocortisone has been demonstrated for 9α-halogenated steroids (1, 2), for compounds methylated at carbon 2 or those possessing both substituents (8), and by 9α-halogenated steroids containing a double bond between carbons 1 and 2 (11). However, each structural alteration alone or in combination greatly accentuated their ability to induce sodium retention.

The present report indicates that the 16α-hydroxylation of 9α-fluoro-Δ^1-hydrocortisone produced no major alteration of its glucocorticoid activity (11), yet completely negated its sodium-retention property. Triamcinolone not only failed to induce water and sodium retention, but actually acted as both a diuretic and natriuretic agent in the saline-loaded, adrenalectomized rat.

A further consideration of these data illustrates the potency of triamcinolone as a glucocorticoid. Subcutaneously administered triamcinolone revealed potencies relative to hydrocortisone and prednisolone of 10 to 40, and 3 to 12, respectively. However, orally, the relative potencies were 41 and 84, and 9 to 19, respectively. This enhanced oral-to-parenteral activity ratio also has been reported by Spero *et al.* (32) for the fluorinated 6-methyl steroids, and by Lyster *et al.* (10) for prednisolone and its 6-methyl analog. Though at present there are no experimental data to explain this phenomenon, these variances may reflect differences in the rates of absorption and/or metabolism of synthetic glucocorticoids.

Life maintenance and growth studies showed that hydrocortisone was less effective than DOC in maintaining the life of adrenalectomized rats, while triamcinolone and prednisolone were at least as effective. Both DOC and prednisolone were superior to triamcinolone in increasing the weight gain of these animals.

While it is difficult to make definite conclusions from these data concerning the relationships of the physiological activity of these steroids to the growth and longevity of adrenalectomized rats, some inferences can be drawn. Using triamcinolone as an example, it is quite apparent that liver-glycogen deposition and sodium excretion are not necessarily adverse to increased longevity in rats. Borman *et al.* (33) present evidence, using halogenated corticoids, that there is not always a direct correlation between sodium-retaining activity and growth-survival activity. 9α-bromo-hydrocortisone acetate, which has little sodium-retaining activity, markedly stimulated growth and survival, while 9α-fluoro-hydrocortisone acetate, which is more potent than DOC as a sodium retainer, and also possesses strong glucocorticoid activity (2), appeared less effective than DOC in growth-survival tests. To further complicate this picture, species differences between rats (33) and dogs (34) have been reported in life-maintenance studies with 9α-halogenated corticoids. Thus, it is apparent that, though salt conservation may be a predominant factor in life maintenance of adrenalectomized rats, protein-carbohydrate metabolism may play a role of considerable importance. This does not preclude the entrance of other, as yet unknown, factors.

A number of structural alterations in the steroid nucleus have been studied in relation to anti-inflammatory activity as measured by granuloma inhibition (35). The presence of various hydroxyl groups (11β, 17α, 21) appears essential for maximum anti-inflammatory potency. Fluorination of the 9α position of hydrocortisone further increased anti-inflammatory activity. In the cotton pellet anti-inflammatory assay Dulin (36) has reported that Δ^1-9α-fluoro-hydrocortisone is 14 times more active than hydrocortisone. Data obtained from the asbestos-pellet assay employed here indicate that triamcinolone is 5 to 10 times more active than hydrocortisone acetate. Since no direct comparison of triamcinolone and Δ^1-9α-fluoro-hydrocortisone was attempted, it is not possible at present to apply significance to the apparent depression in anti-inflammatory activity resulting from 16α-hydroxylation.

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EFFECTS OF RESTRICTED FOOD INTAKE AND GROWTH HORMONE ON RAT LIVER PROTEINS AND NUCLEIC ACIDS[1]

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ABSTRACT

A study was made to determine the part played by food intake on differences observed in rat liver cells of hypophysectomized and growth hormone treated hypophysectomized rats. It was found that: (1) neither restricted food intake nor growth hormone has any effect on the average DNA content of liver nuclei within a given class of ploidy; (2) restricted food intake does play some part in the degree of ploidy shift in the intact as well as in the growth hormone treated hypophysectomized animals. In order to obtain the full shift, both the presence of the pituitary (growth hormone) and an unrestricted food intake are necessary; (3) the inhibitory effect of restricted diet and the stimulatory effect of growth hormone on nuclear and cytoplasmic RNA and protein have been shown.

A differential effect of hypophysectomy on decreased nuclear RNA between diploid and tetraploid nuclei has been demonstrated, and a possible explanation for this effect has been offered.

An attempt has been made to relate these findings to the amino acid transfer hypothesis of growth hormone action.

THE results of a series of studies from this laboratory (1, 2, 3), on livers of young hypophysectomized rats, may be summarized as follows: (1) the amount of deoxyribose nucleic acid (DNA) per nucleus is unaltered by loss of the anterior pituitary hormones or by replacement therapy with a purified growth hormone preparation.[2] (2) The amount of nuclear and cytoplasmic protein and ribose nucleic acid (RNA), as well as nuclear and cytoplasmic volumes are lower than in intact controls of the same age and sex, while treatment of hypophysectomized animals with a purified growth hormone preparation results in a restoration to levels observed in intact controls. (3) There is an arrest of the "ploidy shift," which normally occurs during the growth of the animal, coupled with a marked reduction of mitotic activity. This results in a ploidy pattern like that found at the

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[2] The purified growth hormone (Lot No. M108) was generously supplied by the Endocrine Research Division of Armour and Company. Their analysis of this preparation indicated a complete lack of ACTH and gonadotrophic activities and the presence of a barely detectable TSH activity. The lack of gonadotrophic and ACTH activities was confirmed in our laboratory by the lack of increase in adrenal and testes weights in mg./100 gm. body wt. of hypophysectomized animals treated with the preparation as compared to operated controls treated with saline.

time of hypophysectomy. Growth hormone replacement therapy restores both ploidy distribution pattern and mitotic activity to those of intact controls of comparable weight and sex.

Low protein diets and starvation for varying periods of time have been reported to reduce cell basophilia and to cause other alterations in the chemical morphology of liver cells (4, 5, 6, 7, 8). Therefore, it was pertinent to determine whether or not differences in food intake between growth hormone treated and untreated hypophysectomized rats could be responsible in part for the changes previously reported by us.

MATERIALS AND METHODS

Hypophysectomized and intact male Sprague-Dawley rats were obtained one day after operation from the Hormone Assay Laboratory, Chicago, Illinois. The animals were observed for one day and then divided into 6 groups. The experimental design can be seen best by examination of the following table (Table 1):

Table 1. Experimental design

Group No.	No. of rats	Description	Time of tissue sampling
I	6	Initial intact	Mean body wt. as in II
II	5	Initial hypophysectomized	2 days post-op.
III	10	Intact, fed *ad lib.* for 7 days	8th day
IV	18	Hypophysectomized, fed *ad lib.* for 7 days,	8th day
V	7	Hypophysectomized, 0.25 mg. b.d. G.H. i.m., 7 days, pair-fed with IV	8th day
VI	13	Intact, pair fed with IV for 7 days	8th day

All animals were fed a measured amount of synthetic diet[3] adequate to support normal growth in intact rats of the age, sex and weight range used in these experiments (10 controls, average body weight 90 gm., fed the above diet for 7 days gained an average of 43 gm.).

The animals were sacrificed by decapitation; the livers were rapidly dissected out, blotted, weighed and samples for microspectrophotometric analysis were fixed in 25% neutral formalin. The remainder of the liver was then frozen in alcohol-dry ice and stored at −20° C for further biochemical analyses.

Cytochemical analyses were carried out on 10 μ paraffin sections. The cytophotometric apparatus employed for measurements of protein and nucleic acids *in situ* was a modification of one described by Pollister and Moses (9). The photometer used was a Photovolt Electronic Multiplier Photometer (Model 520M with a 21c search unit).

Determinations of DNA were made by measuring extinctions at 560 mμ of cores, 3 μ in diameter, through the centers of nuclei stained by means of the Feulgen reaction (14 min. hydrolysis in 1N HCl at 60° C, followed by staining with Schiff reagent prepared according to the directions of Stowell (10)). These extinction values were then converted to arbitrary units of DNA per nucleus according to the method described by Swift (11). Whole nuclei were selected at random for studies of ploidy distribution. For each group of experimental animals, the mean DNA content per diploid (2n) and tetraploid (4n) class of nuclei was calculated from frequency distribution plots of all nuclei measured.

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[3] Casein 22%, Wesson oil 5%, Glucose 69%, Salts 4%. Vitamins/100 gm. of diet: Choline 50 mg., Paraaminobenzoic acid 2 mg., Inositol 50 mg., Thiamine 0.5 mg., Riboflavin 1.0 mg., Niacin 2.5 mg., Pyridoxine 0.5 mg., Calcium Pentothenate 5.0 mg., Biotin 0.4 mg., Menodione 0.5 mg., Folic acid 0.2 mg., Vit. B_{12} 12 μg., Vit. A 1,000 I.U., Vit. D 100 I.U., Alpha tocopherol acetate 10 mg.

RNA studies were conducted utilizing basic staining in conjunction with ribonuclease digestion. Sections were digested for 3 hrs. at 37° C in a 0.1% aqueous solution of the enzyme (Armour and Co., Lot 9044X) which had been boiled to remove any traces of proteolytic activity and adjusted to pH 6.0 with NaOH. Control sections were similarly treated except for the absence of the enzyme from the incubating medium. Both control and digested sections were then stained with Azure A (National Aniline Certification No. NAz14) as described by Flax and Pollister (12) and Pollister (13). Thus nuclear and cytoplasmic RNA were computed by subtracting the amount of dye bound after ribonuclease digestion from that found in control sections.

For protein studies, the trichloracetic Millon reaction described by Pollister and Ris (14) was employed.

Extinction measurements of 3 μ plugs through nuclei and cytoplasm were made at 575 mμ for Azure A stained sections and 365 mμ for Millon stained sections. These measurements were made in cells whose nuclear size limits were calculated[4] to insure choosing cells which would be known to fall in each of the two ploidy classes studied (2n and 4n). Since the thickness of the sections used was greater than that of nuclei measured, nuclear extinctions were corrected for the extinction due to cytoplasm in the light path above and below the nucleus. These figures were then converted to arbitrary units of nuclear content as before (11). No attempt was made in these experiments to convert the extinctions measured for cytoplasmic plugs into units of content. These were utilized as extinction values, representing a measure of cytoplasmic concentration rather than content.

The desired wavelengths of light for measurement of extinctions of Azure A and Feulgen stained sections were isolated from a tungsten source by means of a 250 mm. Bausch and Lomb grating monochromator, while for Millon protein determinations the desired wavelength was isolated from a mercury vapor source.

Student's "t" test was used to test for differences between group means at the 1% level. "t" values were calculated from the total number of measurements on individual cells within each experimental group.

RESULTS

Body Weight: The average body weights of the 6 groups of animals at the time of sacrifice were as follows: *Group I*, initial intact, 96 gm.; *Group II*, initial hypophysectomized, 89 gm.; *Group III*, intact fed *ad lib.* for 7 days, 133 gm. (an average gain of 43 gm.); *Group IV*, hypophysectomized fed *ad lib.* for 7 days, 85 gm. (an average loss of 7 gm.); *Group V*, growth hormone treated hypophysectomized pair-fed against IV for 7 days, 110 gm. (an average gain of 14 gm.); *Group VI*, intact pair-fed against IV for 7 days, 90 gm. (an average gain of 6 gm.). The food intake of hypophysectomized animals, and therefore of animals pair-fed against them, was from 35 to 40% of that eaten by the *ad lib.* fed intact controls.

DNA and Ploidy Distribution: Table 2 summarizes the microspectrophotometric data of DNA content of 2n and 4n nuclei and the per cent distributions of these two classes. In all groups, 2n figures clustered around 9 DNA units and 4n figures around 18 DNA units. It is also apparent that, while there was no change in DNA content within a given class of ploidy,

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[4] From ploidy distribution data by determining the ranges of nuclear diameters at which no overlap between 2n and 4n DNA values was observed.

TABLE 2. DNA CONTENT AND PLOIDY DISTRIBUTION OF RAT LIVER CELLS

Group No.	No. of cells	Content*		Per cent distribution*	
		2n	4n	2n	4n
I	400	9.31 ±0.08	18.2 ±0.2	56.3 ±1.5	43.7 ±1.5
II	400	10.1 ±0.1	19.3 ±0.2	62.5 ±1.4	37.5 ±1.4
III	400	9.81 ±0.08	19.2 ±0.2	37.5 ±1.2	64.3 ±1.2
IV	800	9.54 ±0.04	18.9 ±0.1	69.2 ±0.8	30.8 ±0.8
V	400	9.54 ±0.07	18.6 ±0.2	45.1 ±1.3	54.9 ±1.3
VI	400	9.13 ±0.09	18.0 ±0.2	42.6 ±1.1	57.4 ±1.1

* Each figure represents a mean value for the group ±S.E.

alterations in the per cent distributions of the two classes did occur. Diploid nuclei predominated in the ploidy distribution pattern in groups I, II, and IV, while tetraploid nuclei predominated in groups III, V, and VI.

RNA: The data on nuclear and cytoplasmic RNA measurements are presented in Table 3. A differential response of the RNA content in 2n as opposed to 4n nuclei was observed. This was not apparent in the cytoplasm of these two classes of cells. Attention is directed to the fact that in animals 2 days after hypophysectomy the nuclear content and cytoplasmic concentration was below that of initial intact rats (I *vs* II, $P < 0.01$); and that both were further decreased during the following 7 days (II *vs* IV, $P < 0.01$). The nuclear content and cytoplasmic concentration remained essentially unchanged in intact rats allowed food *ad lib.* for the 7 day experimental period (I *vs* III, $P > 0.1$). In pair-fed intact animals, during the same period of time, both nuclear content and cytoplasmic concentration of RNA were less than that of the *ad lib.* fed (III *vs* VI, $P < 0.01$). This decrease however, was not of the same magnitude as that seen in untreated hypopophysectomized animals (IV *vs* VI, $P < 0.01$).

Protein: The results of the protein studies are shown in Table 4. Differences in protein may be seen in both the nucleus and cytoplasm of the two ploidy classes of cells studied. Within each ploidy class the per cent change in all groups was the same for both nuclei and cytoplasm. Except for the lack of a differential response in protein between 2n and 4n nuclei which was observed in the RNA studies, the protein data paralleled the RNA data.

TABLE 3. NUCLEAR AND CYTOPLASMIC RNA OF RAT LIVER CELLS

Group No.	No. of cells	*Nucleus*				Cytoplasm			
		Content*		Per cent of initial intact		Extinction*		Per cent of initial intact	
		2n†	4n‡	2n	4n	2n†	4n‡	2n	4n
I	200	4.95± .09	7.92± .10	100	100	.426± .015	.451± .018	100	100
II	200	3.19± .06	6.05± .08	64	76	.255± .011	.257± .012	60	61
III	200	5.17± .08	8.25± .11	104	104	.439± .019	.473± .015	103	105
IV	400	2.64± .03	5.90± .04	53	75	.217± .008	.223± .007	51	50
V	200	4.42± .09	6.72± .09	89	85	.325± .012	.332± .013	76	74
VI	200	3.96± .08	6.55± .10	80	83	.335± .012	.368± .015	79	75

* Each figure represents a mean value for the group ±S.E.

† Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 5.5 and 6.0 μ.

‡ Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 7.8 and 8.3 μ.

TABLE 4. NUCLEAR AND CYTOPLASMIC PROTEIN OF RAT LIVER CELLS

Group No.	No. of cells	Nucleus				Cytoplasm			
		Content*		Per cent of initial intact		Extinction*		Per cent of initial intact	
		2n†	4n‡	2n	4n	2n†	4n‡	2n	4n
I	200	6.21 ± .11	13.3 ± .15	100	100	.640 ± .008	.615 ± .008	100	100
II	200	5.64 ± .10	11.9 ± .14	90	89	.572 ± .009	.553 ± .008	89	90
III	200	6.41 ± .09	14.0 ± .15	103	105	.667 ± .011	.648 ± .010	104	105
IV	400	4.43 ± .06	10.1 ± .10	71	76	.465 ± .005	.433 ± .004	73	70
V	200	5.73 ± .09	12.4 ± .12	92	93	.581 ± .009	.560 ± .008	91	91
VI	200	5.54 ± .09	12.1 ± .16	89	91	.585 ± .008	.569 ± .009	91	93

* Each figure represents a mean value for the group ± S.E.

† Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 5.5 and 6.0 μ.

‡ Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 7.8 and 8.3 μ.

DISCUSSION

Under conditions of these experiments neither growth hormone nor restricted food intake influenced the DNA content of rat liver cells within a given class of ploidy. This is in agreement with our previous findings (1, 2, 3) and with the findings of other workers who, by means of other techniques, have shown a lack of dietary effects on the amount of DNA within a liver cell nucleus (15).

In an earlier publication Di Stefano, *et al.* (3), in livers of rats of the same strain, weights and sex as those used in these experiments, reported a "ploidy shift" from a predominance of 2n nuclei to a predominance of 4n nuclei in intact animals fed *ad lib.* for 7 days; an inhibition of this "ploidy shift" in the hypophysectomized rat; and a restoration of ploidy patterns to control levels following growth hormone replacement therapy of hypophysectomized animals allowed to eat *ad libitum.* These observations have been confirmed and extended in the present study. Our earlier conclusion for this failure of development of the "ploidy shift" was that the lack of growth hormone was responsible for the maintenance of the more juvenile state of ploidy characteristic of the liver at the time of hypophysectomy. In this respect the synthetic activities of the cell necessary for the cell division, which must be a prerequisite for a change from a 2n to a 4n predominance, would be markedly reduced or inhibited by the lack of the anterior pituitary.

The data presented in this paper indicate that the "ploidy shift" was significantly retarded in the pair-fed intact rats (compare groups III and VI, Table 2, P <0.01). In the hypophysectomized animals given growth hormone while pair-fed against *ad libitum* fed hypophysectomized animals, the resultant ploidy pattern was the same as that observed in the pair-fed intact rat. That the major portion of the ploidy shift is growth hormone dependent is indicated by a comparison of the observed growth hormone effect (groups IV, V, and VI, Table 2; V *vs* VI, P >0.1; IV *vs* V and/or VI, P <0.01) with the dietary restriction effect referred to above. The order of magnitude of the hormone effect is clearly greater than the inhibitory effect of diminished food intake.

Dietary effects resulting in losses of liver tissue components following periods of fast or of feeding protein deficient diets have been reported by several investigators. Kosterlitz (5) has reported the loss of 27% of the liver cytoplasm in rats restricted to a protein-free diet for a period of 7 days; Brachet, *et al.* (6), who compared fasted and non-fasted control mice, noted a marked reduction in cytoplasmic protein and RNA in the livers of animals fasted for 4 days; Lagerstedt (7) studied changes in rat liver cells produced by the quantitative variations in the protein content of the diet, ranging from complete starvation for a period of 5 days through feeding of high protein diets to starved animals, and found that in fasted animals, as well as in animals on low protein diets, the degree of nuclear basophilia was markedly reduced and that of the cytoplasm eventually was completely lost. These parameters, he found, could be reversed by restoring protein to the animal's diet. Thomson, *et al.* (8) reported that in rat liver, a 48 hour fast caused a reduction of lipid phosphorus, protein nitrogen, and RNA by 20 to 30%. Our findings are in agreement with those of these workers since we observed a decrease of the same order of magnitude in these parameters in pair-fed animals whose food intake was reduced by some 60% of that of *ad lib.* fed intact animals. Although a reduction of nuclear and cytoplasmic RNA and protein was seen in the liver cells of the intact rats whose food intake was restricted by pair-feeding against hypophysectomized rats, this decrease was not as great as that seen in the hypophysectomized animals. This points to the fact that a major portion of the decreased RNA and protein in the liver cells of the hypophysectomized rat was due to the lack of the anterior pituitary and only partially to the lowered food consumption of these animals. That moiety of RNA and protein loss which was due to hypophysectomy was restored to pair-fed intact control levels by growth hormone administration, while both growth hormone treatment and unrestricted food intake would appear to be necessary in order to return these values to normal levels (1, 2).

The mechanism of the differential response to hypophysectomy of the 2n and 4n nuclear RNA mentioned above is not known. Since the age of the animals used in these experiments is such that in the intact rat there is a rapid shift from 2n to 4n predominance, the mitotic rate must be higher in the 2n class in order to allow this shift to take place; therefore, it may be that any deficiencies in the synthesis of RNA are brought into relief in this class.

It must be emphasized once again that a description of the effect of a hormone on the reproductive and protein synthetic apparatus of the cell does not necessarily imply that the biochemical locus of action of the hormone is intimately related to effects described. However, if an understanding of the effect of growth hormone on cells is to be achieved, an attempt to relate the observations described herein to other known actions of the hormone is in order.

Recently Noall, *et al.* (16) have made the intriguing suggestion that growth hormone may have as its primary effect that of concentrative transfer of amino acids intracellularly. Certainly, the lowering of blood amino acid nitrogen that occurs following the administration of growth hormone to the intact animal can be seen within a few minutes (17). In contrast, nucleic acid and protein changes of the type described in this report have not been seen as late as 12 hours after initiation of growth hormone treatment in the hypophysectomized rat (18). Since a number of investigators have suggested that amino acids play a catalytic role in the synthesis of RNA and possibly DNA as well (19, 20, 21) the amino acid concentrative transfer hypothesis becomes progressively more appealing. For, if one postulates that the nucleic acid synthetic activities of cells (and, secondarily, their mitotic activity) are to some extent controlled or regulated by the size of the intracellular free amino acid pool, the RNA and DNA effects of growth hormone we have described could easily be secondary to an effect of growth hormone on some cell membrane amino acid transfer process. The fact that food restriction has an inhibitory effect on RNA synthesis, protein synthesis and mitotic activity may be explained partly on the basis of diminished intracellular amino acid pool size and partly on that of a diminished availability of calories for the energetic requirements of synthetic reactions.

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STUDIES ON THE EFFECT OF STORAGE ON THE PURITY OF SODIUM IODIDE-I^{131}[1]

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ABSTRACT

Solutions of NaI^{131} upon storage show the presence of several bands (usually six inclusive of NaI^{131}) when chromatographed one dimensionally in an ascending technique with various solvent mixtures and radioautographed. The percentage of radioactivity in the bands other than NaI^{131} increases rapidly upon storage at room temperature. Incubation of NaI^{131} solutions under nitrogen for 1½ hours with 40 mg./ml. of cysteine at pH 4 or pH 7 or 10 mg./ml. of NaI^{127} or KI^{127} at pH 7 converges the extraneous bands into the NaI^{131} band. Preliminary studies rule out the possibility that the bands other than NaI^{131} are due to oxides of iodine or due to iodides other than NaI^{131}. Studies on the uptake of the NaI^{131} or the major extraneous band by day old chick thyroids indicate that 20% of the injected radioactivity of the extraneous band is collected in the thyroid in contrast to the 60% uptake of NaI^{131}.

SODIUM iodide-I^{131} (NaI^{131}) is widely used for thyroid metabolism studies. Taurog, Potter and Chaikoff reported (1) that radioiodide obtained from Oak Ridge shows faint extraneous bands upon chromatography and that addition of thiouracil, thiosulfate or sulfite eliminates their presence. Sodium iodide-I^{131} which is used for clinical studies[2], also shows several bands when chromatographed one dimensionally in an ascending technique using several different solvent systems. The precentages of radioactivity in the bands other than NaI^{131} increases rapidly upon storage. The present study is concerned with the effect of storage on the purity of NaI^{131} and with the factors influencing the conversion of the various extraneous components into NaI^{131}. Preliminary studies are reported on the nature of the bands other than NaI^{131}.

METHODS

Chromatography and Radioautography

NaI^{131} (Abbott Radio-Pharmaceuticals) after storage at room temperature for four weeks was spotted in 0.6 μc. amounts on Whatman No. 1 filter paper strips and developed

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[2] Furnished by Abbott Radio-Pharmaceuticals, Oak Ridge, Tennessee or by E. R. Squibb & Sons, New Brunswick, New Jersey.

one dimensionally in an ascending technique using the following solvent mixtures: (A) n-Butanol saturated with 3N NH_3OH; (B) n-Butanol:acetic acid:water::78:5:17; (C) Wet collidine; (D) isoamyl alcohol saturated with 2N NH_4OH; (E) n-Butanol:dioxane:2N NH_4OH:50:12.5:37.5; (F) Methanol:water::70:30. After 20 hours, the strips were air dried and radioautographed. The location of the NaI^{131} band on the radioautograms was determined by running control strips spotted with a solution of 0.5 mg. NaI^{127} and developed under the same conditions. The NaI^{127} band was detected by dipping the strip in a solution of ammoniacal silver nitrate. The various bands obtained after chromatography and radioautography of NaI^{131} solution using solvent A were cut out, eluted with 50% ethanol and the radioactivity in each band was measured in a well counter. The percentages of the radioactivity in the bands from the origin upwards were 19, 21, 16, 32, 4 and 8% respectively. Since solvent A gave the best separation of the extraneous bands from NaI^{131}, this solvent system was used throughout the present study.

Effect of Storage of NaI^{131} on the Conversion of NaI^{131} into the Extraneous Bands

Three separate batches of NaI^{131} solutions which were 1, 4 and 7 weeks old were chromatographed using solvent A and radioautographed. The results are shown in Figure 2. In a separate experiment, solutions of NaI^{131} were stored from 1 to 4 weeks under sterile conditions and also in a frozen state.

Factors Affecting Conversion of the Extraneous Bands into NaI^{131}

Solutions of NaI^{131} (4 weeks old) containing 40 mg./ml. of thiouracil, cysteine, homocysteine, glutathione or ascorbate were incubated under nitrogen at 37° C for 1½ hours. At the end of the incubation time an aliquot was spotted on Whatman No. 1 paper strips and chromatographed with solvent A and radioautographed. The results are shown in Figure 3. Glutathione and cysteine converge the extraneous bands into NaI^{131} as shown in Figure 3. The effect of pH on the ability of glutathione or cysteine to converge the extraneous bands into NaI^{131} was studied. The results are presented in Table 1. The recovery of radioactivity of the extraneous bands converged into the NaI^{131} band following incubations with cysteine (pH 7) was determined. The effect of different levels of NaI^{127} and KI^{127} in converging the extraneous bands from NaI^{131} solutions into NaI^{131} was also studied.

TABLE 1. EFFECT OF pH ON THE CONVERSION OF EXTRANEOUS BANDS INTO NaI^{131} BY CYSTEINE AND GLUTATHIONE

Substances incubated*	pH of the solutions	Distribution of radioactivity % in bands					
		1	2	3	4	5	6
NaI^{131} control	4	24	16	11	47	1	1
	7	24	17	11	45	2	1
	10	24	17	13	44	1	1
NaI^{131}+cysteine	4	1	0	97	0	1	1
	7	1	0	98	0	1	0
	10	9	1	72	18	0	0
NaI^{131}+glutathione	4	1	0	99	0	0	0
	7	5	1	85	7	1	1
	10	11	2	61	24	1	1

* Solutions of NaI^{131} (44 days old) containing 40 mg./ml. cysteine or the same amount of reduced glutathione were adjusted to pH 4, 7 or 10 and incubated under nitrogen at 37°C for 1½ hours. Aliquots containing 0.6 μc. of incubated material were chromatographed using solvent A, and radioautographed. The different bands were cut out, eluted and counted.

Studies on the Identity of the Extraneous Bands

Chromatograms were run using solvent A on NaI^{127} solutions or solutions of mixtures of NaI^{127} with ascorbate or bisulfite which were stored for 4 weeks. NaI^{127} band was identified by dipping the chromatograms in ammoniacal silver nitrate. The Rf. values of KI^{127}, $ZNI_2{}^{127}$, $RbI_2{}^{127}$ and $MgI_2{}^{127}$ with solvent A were found to be the same as that of NaI^{127}. The Rf. values of sodium iodate, sodium meta-periodate and sodium periodate with solvent A were determined. Effect of autoclaving on NaI^{127} or of x-irradiation (2,000γ) on NaI^{127} solution with regard to the presence or absence of extraneous bands was studied.

RESULTS AND DISCUSSION

The radioautographs of chromatograms of NaI^{131} solutions (4 weeks old) developed with the various solvent mixtures clearly indicate that upon storage, NaI^{131} solutions contain more than one band (Fig. 1). Solvents F and B do not resolve the different bands effectively but when developed with solvents A, C, D and E, NaI^{131} solutions separate into 5 or 6 bands. Of these, the NaI^{131} band is number 3 solvent A, number 2 with solvent B, number 4 with solvent C, number 2 with solvent D, number 4 with solvent E and number 3 with solvent F. The rest of the bands are extraneous components, probably formed from NaI^{131} on storage. The percentages of the total radioactivity in the six bands obtained by chromatographing NaI^{131} solutions with solvent A were 19% in band 1, 21% in band 2, 16% in band 3 (NaI^{131} band), 32% in band 4, 4% in band 5 and 8% in band 6 (Fig. 1, solvent A). Thus only 16% of the total radioactivity is in the NaI^{131} band while the rest is distributed among five other components.

The results shown in Figure 2, in which NaI^{131} solutions were stored at room temperature for different periods indicate clearly an increase in the radioactivity of the extraneous bands on storage. This was also true of NaI^{131} solutions stored under sterile conditions or in a frozen state. Since Na^{131} solutions are used extensively both clinically and for research purposes, it is extremely important to determine the identities and properties of the various extraneous substances formed in solutions of NaI^{131}. The NaI^{131} solutions which are freshly received or which are only a week old also contain a significant amount of radioactivity in band 4 as shown in Figure 2.

Of the several reducing agents tested (thiouracil, cysteine, homocysteine, glutathione and ascorbate) for their ability to converge the extraneous bands into NaI^{131}, cysteine and glutathione gave the best conversion as shown in Figure 3. That this is true "conversion" and not merely a disappearance of the extraneous substances was shown as follows: The radioactive substances making up the various bands both in the control strip and in the strip in which cysteine had converged the bands were eluted and counted. The radioactivity in the NaI^{131} band from the cysteine strip was 90% of the total counts of the individually eluted bands from the

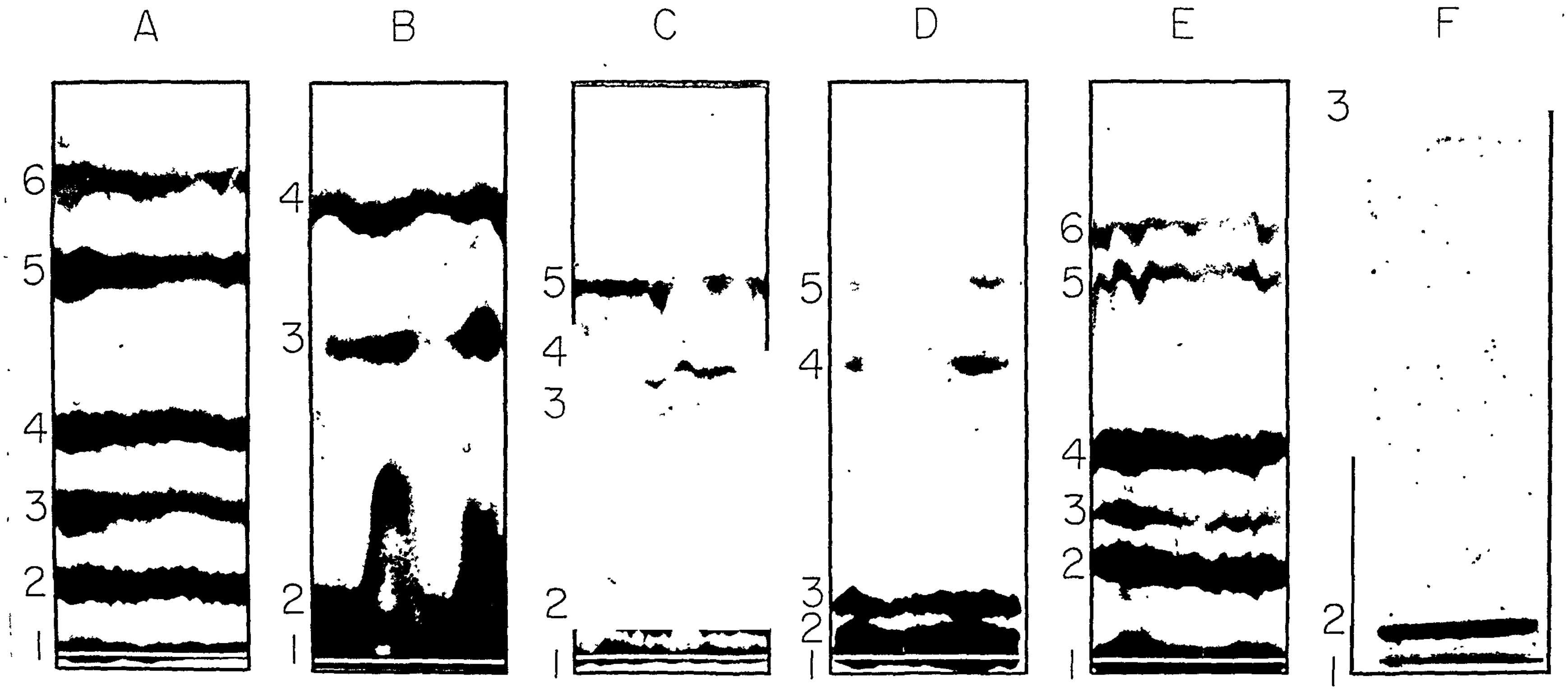

FIG. 1. Radioautographs obtained from chromatograms of NaI^{131} solutions (4 weeks old) developed with the following solvent mixtures: (A) n-Butanol saturated with 3N NH_4OH; (B) n-Butanol:acetic acid:water::78:5:17; (C) Wet collidine; (D) Isoamyl alcohol saturated with 2N NH_4OH; (E) n-Butanol:dioxane::4:1 saturated with 2N NH_4OH; (F) Methanol:water::70:30. The NaI^{131} bands are nos. 3, 2, 4, 2, 4, and 3 from the origin with solvents A through F respectively. The percentages of the total radioactivity in the different bands obtained with solvent A are as follows: bands 1 through 6 are 19, 21, 16, 32. 4 and 8% respectively.

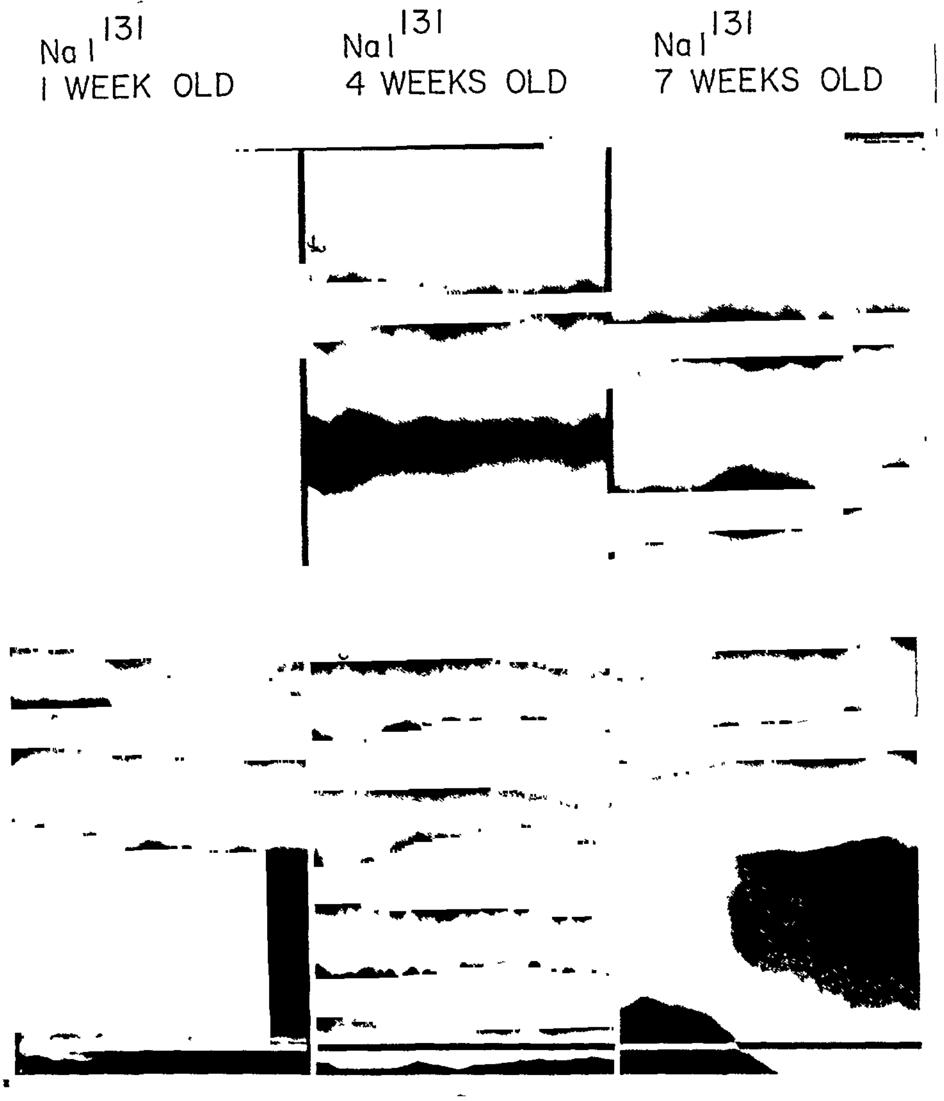

FIG. 2. Effect of storage of NaI^{131} solutions on the conversion of NaI^{131} into extraneous bands. Strips 1, 2 and 3 represent radioautographs of chromatograms of NaI^{131} solutions from batches which are 1, 4 and 7 weeks old respectively. The chromatograms were developed using solvent A.

control strip. The results of the study on the effect of pH on the ability of cysteine and glutathione to converge the extraneous bands into NaI^{131} are presented in Table 1. Eleven to 13% of the total radioactivity of NaI^{131} solutions is present in the NaI^{131} band, while the remaining 87% is distributed among 5 extraneous bands. Changes of pH's do not influence the percentage of the radioactivity in the NaI^{131} band in the control experiment. At pH 4, both glutathione and cysteine are effective in converging the extraneous bands into NaI^{131}. However, at pH 7, cysteine is superior to

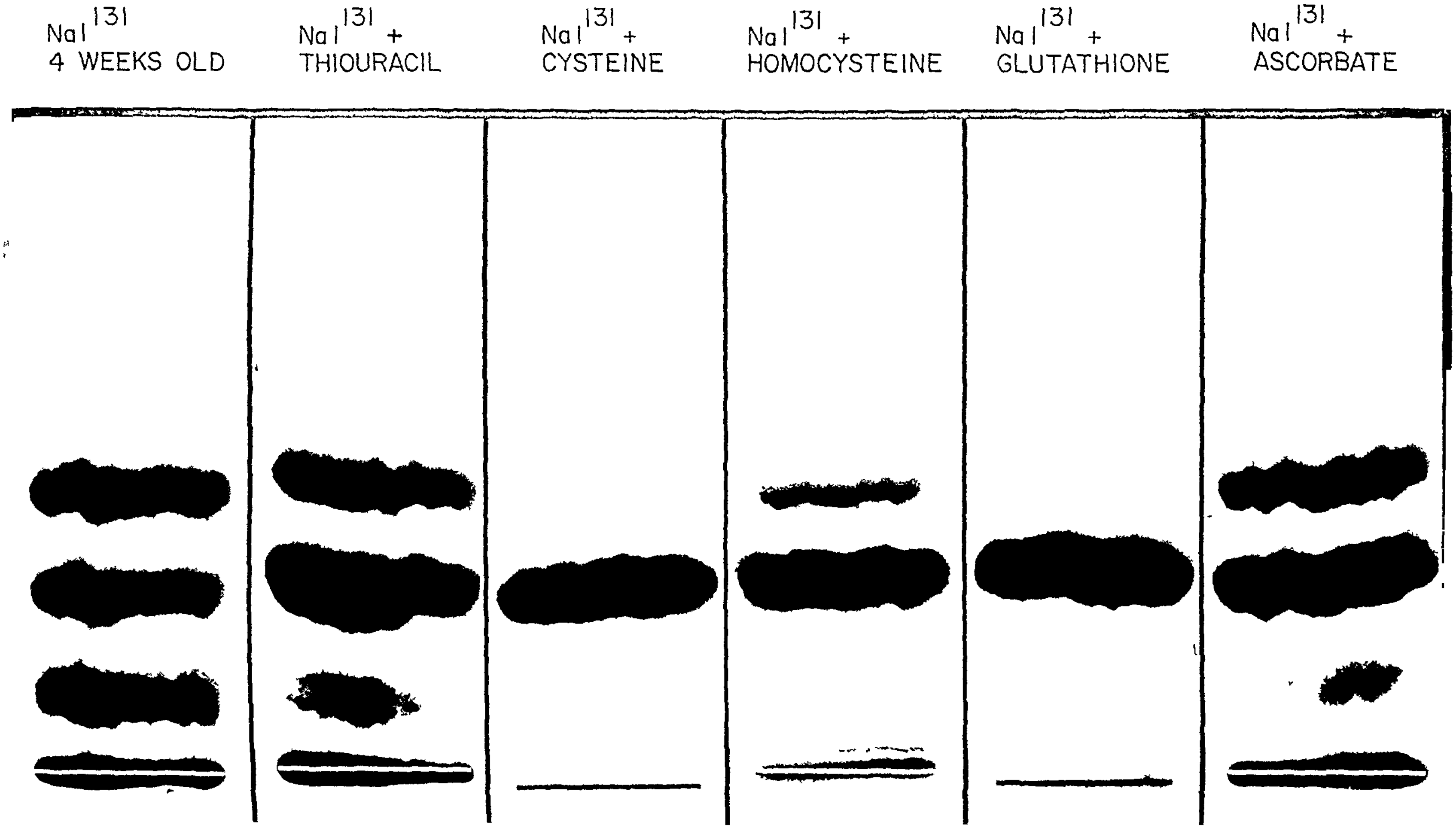

FIG. 3. The effect of reducing agents on the conversion of extraneous bands into NaI^{131}. Solutions of NaI^{131} (4 weeks old) containing 40 mg./ml, of thiouracil, cysteine, homocysteine, glutathione and ascorbate were incubated under nitrogen at 37° C for 1½ hours. An aliquot containing 0.6 μc. of the incubated material was chromatographed with solvent A and radioautographed.

glutathione. On the other hand, at pH 10, both cysteine and glutathione are less effective than at pH 4 or at pH 7.0. Incubations of NaI^{131} solutions with 10 mg./ml. of NaI^{127} or KI^{127} at pH 7.0 for $1\frac{1}{2}$ hours also effectively converges the extraneous bands into NaI^{131} band.

Aqueous solutions of NaI^{127} (1%) which have been stored at room temperature for 4 weeks still showed only one NaI^{127} band when chromatographed with solvent A. Addition of ascorbate or bisulfite to the NaI^{127} (1%) solutions do not affect the NaI^{127} band. Iodides with different cations (K, Zn, Rb and Mg) have the same Rf. value as the NaI^{127} band with solvent A. None of the extraneous bands observed in NaI^{131} solutions have the same Rf. values with solvent A as those of sodium iodate, sodium metaperiodate or sodium periodate. Solutions of NaI^{127} (1%) which are autoclaved for 15 minutes at 120° C give only one NaI^{127} band when chromatographed with solvent A. X-irradiation (2,000γ) of a solution of NaI^{131} does not increase the number or quantity of the extraneous bands. These studies rule out the possibility that the extraneous bands found in NaI^{131} solutions are iodides with different cations or that they are oxides of iodine.

Studies were conducted using day old chicks (New Hampshire males × Single Comb White Leghorn females) to determine the uptake of NaI^{131} or the major extraneous band (band 4, Fig. 1, Strip A) by the thyroid. Several microcuries of NaI^{131} solutions (4 weeks old) were spotted on sheets of Whatman No. 1 filter paper and developed using solvent A and radioautographed. The extraneous band (No. 4, Fig. 1, Strip A) and NaI^{131} band were eluted from the chromatograms and evaporated under nitrogen to 10 μc./ml. concentration. Six chicks were injected in the breast muscle with 1 μc. each of the NaI^{131} and three were given 1 μc. each of the extraneous band. At the end of 24 hours, the animals were killed by decapitation, the thyroids were removed and the collection of radioactivity by the thyroids was determined using a well counter. The results indicate that the three chicks which received the extraneous band collected only 20% of the injected radioactivity, while the six chicks injected NaI^{131} collected on the average 60% of the injected dose.

Reports of further studies of the biological properties of each band will appear in a separate communication.

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NOTES AND COMMENTS

THYROXINE EXCRETION INCREASE BY COLD EXPOSURE[1]

ABSTRACT

Rats were maintained in a cold room at 10° C for 11 days. After injection of potassium perchlorate and I^{131} thyroxine, excretory depletion rates of radiothyroxine were measured for a 4 day period. During the first twenty-four hours, cold-exposed rats excreted twice as much fecal I^{131} as rats at room temperature. This suggests that the goitrogenic effect of cold may be related to thyroxine depletion by an increased rate of fecal excretion.

Fecal iodine is predominantly organic iodine (1) *derived from the thyroid gland* (2). Fecal excretion of I^{131} thyroxine has been reported to be greater in rats fed a high concentration of dietary bulk as compared to rats fed diets relatively low in bulk (3). This has been related to an interference with reabsorption of biliary thyroxine (3, 4). Reichlin (5) reported that starvation in rabbits decreased fecal I^{131} thyroxine excretion which accompanied decreased fecal volume. Since cold-exposure increases thyroxine requirement (6), thyroid size (7), and food consumption (8), it was reasoned that the increased fecal volume associated with the increase in food consumption may alter the loss of fecal thyroxine in cold exposed animals.

METHODS

A group of albino rats was exposed to 10 ± 0.5° C for 11 days, another group was kept at 22 ± 1° C as controls. Purina dog chow (iodine content 1.4 μg./gm.) was fed *ad libitum*. The control rats ate approximately 15 gm. of food per day and those at 10° C ate 25 gm. per day. This resulted in an iodine intake which was at least 10 times greater than the requirement. Both groups were injected intraperitoneally with 5 mg. potassium perchlorate solution. Thirty minutes later they were similarly injected with I^{131} L-thyroxine[2] (1.1 μg., 2.3 μc. in .05 cc. of propylene glycol). Potassium perchlorate (5 mg./day) was administered continuously in the diet for 4 days. The animals were kept in wire-bottom metabolism cages and daily excretion collected separately for 4 days after thyroxine injection. Thyroids were blocked with potassium perchlorate in preference to other antithyroid agents because Van Arsdel has suggested (9) that propylthiouracil increased fecal excretion of radiothyroxine but perchlorate did not alter the metabolism of the hormone. After radiothyroxine and perchlorate injections, synthesis of endogenous thyroxine was minimized and the body content of thyroxine began to be depleted. Iodine reserves were measured (11) in plasma and thyroid of similar rats not administered potassium perchlorate and thyroxine.

Fecal I^{131} has been shown to be predominantly thyroxine after radiothyroxine injection (1), therefore the rate of fecal excretion of I^{131} measured the excretory depletion rates of thyroxine from animals.

RESULTS

Table 1 shows the iodine reserves were similar in rats exposed to room temperature and the mildly cold environment of 10° C. Table 2 shows that during the first 24 hours, rats at 10° C excreted about twice as much fecal thyroxine as controls. After one day, the cold-exposed rats retained only 13% of the administered dose compared to 43% retention in the controls. Initial urinary radioiodide appeared to be greater in rats at 10° C but the large standard deviations prevented these urinary data from showing statistically significant differences. Fecal excretion of I^{131} thyroxine was not significantly different at the two temperatures during the second day. The fecal and urinary I^{131} excreted during the

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[2] Prepared by Abbott Laboratories.

TABLE 1. IODINE RESERVES OF CONTROL RATS EXPOSED TO 10° C AND 22° C FOR 37 DAYS (Diet, Purina Dog Chow)

No. of rats	Temp.	Protein-bound iodine	Thyroid iodine	Thyroid wt.
	° C	μg.%	μg.	mg./100 gm. body wt.
8	10°	4.7	13.1	7.9
8	22°	5.6	14.4	7.5

third day after injection was significantly greater in the control group. This reversal of radioiodine excretion is probably due to the relatively large initial retention in the control group. Since the cold-exposed rats lost the greatest percentage of the dose the first day, less radiothyroxine was available for subsequent excretion in the cold as compared to the amount available for excretion in the controls. Accumulative fecal radiothyroxine during the 4-day experimental period was 59.7% at 10° C and 45.9% at 22° C ($p < .02$). The data indicate that after 96 hours both groups have lost only slightly different amounts of organic iodine in the feces, but the animals exposed to 10° C lost it more rapidly. The effectiveness of thyroid blockade by perchlorate was shown by hypertrophy of the glands within 96 hrs. Among these animals the cold-exposed rats developed larger thyroids (18.7 mg./100 gm. body wt.) than the rats at 22° C (11.7 mg./100 mg. body wt.).

TABLE 2. EXCRETION OF I^{131} AFTER INTRAPERITONEAL INJECTION OF I^{131} THYROXINE; RATS EXPOSED TO 10° C AND 22° C

(Thyroids blocked with $KClO_4$)

No of rats	Avg. body weight	Temp	Excreta collection periods (hours after thyroxine injection) 0–24		24–48		48–96		Plasma I^{131} after 96 hrs	Carcass I^{131} after 96 hrs.
			% of thyroxine dose							
	grams	° C	feces	urine	feces	urine	feces	urine	% dose/cc	% dose
8	198	10°	47	40	10.6	7.7	2.1	1.5	.01	0.7
Standard Deviation			± 17.5	± 12.5	± 7.4	± 3.7	± 1.4	± .8	± .005	± .38
7	200	22°	24.5	32	13.9	14	7.5	7.4	.05	3.1
Standard Deviation			± 3.8	± 5.1	± 2.1	± 1.1	± .48	± 1.1	± .018	± .60
p*			< .02	.2	.8	< .005	< .001	< .001	< .001	< .001

* p values from Fisher's t-test (10) applied to differences between means at 10° C and 22° C

DISCUSSION

Rats exposed to 10° C for 11 days and then injected with radiothyroxine excreted I^{131} in the feces at a faster rate than controls at 22° C. This mild cold exposure was not goitrogenic in rats maintained on a high iodine intake. Thus, the increased thyroxine excretion occurring at 10° C can be adequately replenished by a high iodine diet. Animals on a lower iodine intake may not be able to replace this rapid thyroxine loss. Increased fecal thyroxine excretion in the cold should increase thyroxine requirements and thereby may be a major cause of the increased incidence of goiter in animals exposed to moderate cold. We consider that any factor which increases dietary intake and fecal volume might increase fecal thyroxine excretion.

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THE ANTI-ANDROGENIC ACTIVITY OF A PHENANTHRENE DERIVATIVE IN THE CHICK*

ABSTRACT

2-Acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene when inuncted directly on the chick's comb inhibits the stimulating effect of injected testosterone.

Anti-androgenic agents may be evaluated by direct inunction on the chick's comb stimulated by testosterone incorporated into the diet (1). This communication reports the anti-androgenic activity of a nonsteroidal compound, 2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene (Ro-2-7239) by a modification of this assay procedure and confirms the anti-androgenic activity reported for this compound in the castrated rat by Randall and Selitto (2).

METHOD

Two-day old White Leghorn cockerels were injected subcutaneously with 0.5 mg. of testosterone enanthate contained in 0.5 ml. of sesame oil. On the same day and for the following six consecutive days the test compound dissolved in 0.05 ml. of sesame oil was inuncted on the comb once daily. One day after the last inunction the chicks were sacrificed and body weights and comb weights were determined. The results are expressed as comb ratios, defined as mg. of comb per gram of body weight.

RESULTS AND CONCLUSIONS

Norethisterone (1) (19-Nor-17-ethynyltestosterone) in amounts of 0.5 to 1.0 mg. significantly inhibited the stimulating effect of injected testosterone (Table 1). Table 2 illustrates the fact that Ro-2-7239 was approximately as active as norethisterone in inhibiting the action of testosterone. The mean comb ratios of groups of untreated chicks varied between 0.33±0.011 and 0.38±0.017. Thus the maximum inhibitory effects of norethisterone and Ro-2-7239 were 60% and 44% respectively. Ro-2-7239 does not ap-

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TABLE 1. THE ANTI-ANDROGENIC ACTIVITY OF NORETHISTERONE
(All chicks received a stimulating dose of 0.5 mg. of testosterone enanthate)

Test No.	Material inuncted	Total dose, mg.	No. of chicks	Mean comb ratio ±S.E.
1	0	0	44	0.93 ±0.029
	Norethisterone	0.25	37	0.77 ±0.028
		0.50	35	0.73 ±0.025
		1.00	35	0.71 ±0.030
2	0	0	25	1.02 ±0.055
	Norethisterone	0.25	20	0.76 ±0.035
		0.5	20	0.77 ±0.048
		1.0	20	0.64 ±0.025
3	0	0	29	1.23 ±0.072
	Norethisterone	0.25	30	1.18 ±0.066
		0.5	30	0.80 ±0.042
		1.0	30	0.74 ±0.031

TABLE 2. THE ANTI-ANDROGENIC ACTIVITY OF Ro-2-7239 COMPARED TO NORETHISTERONE

Test No.	Material inuncted	Total dose, mg.	No. of chicks	Mean comb ratio ±S.E.
4	0	0	24	1.08 ±0.068
	Norethisterone	0.25	22	0.93 ±0.041
		0.5	21	0.92 ±0.043
		1.0	23	0.85 ±0.041
	Ro-2-7239	0.1	17	0.97 ±0.034
		0.2	17	0.92 ±0.042
		0.4	17	0.81 ±0.042
		1.6	13	0.73 ±0.055
		5.0	12	0.74 ±0.036
5	0	0	28	0.81 ±0.037
	Norethisterone	0.25	23	0.67 ±0.027
		0.50	23	0.65 ±0.031
		1.00	24	0.67 ±0.027
	Ro-2-7239	0.1	19	0.66 ±0.069
		0.4	20	0.63 ±0.025
		0.8	20	0.61 ±0.044
6	0	0	19	1.02 ±0.035
	Norethisterone	0.25	18	0.92 ±0.049
		1.00	18	0.70 ±0.049
	Ro-2-7239	0.25	20	0.69 ±0.050
		1.00	20	0.74 ±0.031

pear to have any other hormonal effects (2) and in this sense is comparable to other non-steroidal anti-androgens including methylcholanthrene (3) and 20-methyl-Δ^5-pregnene-3β,20-diol (4).

Acknowledgments

Thanks are due to Hoffmann-LaRoche, Inc., for the supply of Ro-2-7239, to Syntex S. A., Mexico City, for norethisterone, and to Mr. Albert Tomolonis and Miss Theresa Rutkiewicz for their valuable technical assistance.

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INHIBITION OF THYROXINE ACTION BY 3,3′,5′-TRIIODOTHYRONINE[1]

ABSTRACT

L-Thyroxine injected in doses of 10–20 μgm./kg./d. for 2–3 weeks into thyroidectomized rats maintained the BMR at about 20% above the hypometabolic level. DL-3,3′,5′-Triiodothyronine completely reversed the effect when used at 2 mg./kg./d.

The increased oxygen consumption of tissues removed from thyroxine-injected hypothyroid animals could also be depressed by the concomitant injection of 3,3′,5′-triiodothyronine.

3,3′,5′-Triiodothyronine (3,3′,5′-Trit) has been encountered in the thyroid gland (1) and blood (2) of I^{131}-injected experimental animals. In contrast to its 3,5,3′-isomer, this compound has been found to produce less than 0.5% of the metabolism-stimulating effect of thyroxine (3). Since 3,5,3′-Trit is some four times as active as thyroxine, the 3,3′,5′-Trit can be considered inactive. Therefore it was thought of interest to evaluate its action in inhibiting the metabolic effect of thyroxine, particularly in view of the earlier report by Cortell (4) that 2′,6′-diiodothyronine possessed thyroxine-inhibiting properties.

METHODS

Thyroidectomized Sprague-Dawley rats were used, at least two months after operation. For studies on the BMR of the whole animal, post-absorptive rats on a special feeding regime (5) were trained to the chamber of an apparatus similar to that described by Watts and Gourley (6). After a series of satisfactory control determinations, selected animals were started on L-thyroxine[2] injected daily subcutaneously at 10 or 20 μg./kg. When the elevated BMR values became stabilized, usually after 3 weeks, the animals were given 3,3′,5′-Trit[3] subcutaneously in addition to thyroxine. The level of depressed metabolism was established for several days and the 3,3′,5′-Trit withdrawn, leaving the animals on thyroxine alone.

The tissue slice studies employed conventional Warburg oxygen consumption measurements on representative organs (liver, kidney, salivary glands, diaphragm, and heart) excised from thyroidectomized rats which had been injected for four days with one of the following: blank, thyroxine (0.25 mg./kg./d.), or thyroxine plus varying amounts of 3,3′,5′-Trit, (10 or 20 mg./kg./d.).

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[1] This investigation was supported by research grant A-1545 from the Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service.

[2] L-Thyroxine was furnished by Dr. H. L. Fevold of the Travenol Division, Baxter Laboratories.

[3] 3,3′,5′-Triiodothyronine was provided by Drs. R. I. Meltzer and R. L. Kroc of the Warner-Chilcott Laboratories.

RESULTS

Figure 1 charts the time course of BMR response of a group of 3 thyroidectomized rats. Each point represents the mean oxygen consumption of the 3 animals under the treatment shown. The horizontal lines show the average value over the time limit indicated. For instance, line #1 indicates the control BMR at 88.9 ml. O_2/100 gm. body wt./hr. over the span of 7 days; line #2 at 108.8 ml. O_2, representing the plateau of response to 10 μgm. of thyroxine. When 2 mg. of 3,3′,5′-Trit was superimposed, the metabolic rate decreased to line #3, 86.2 ml. O_2 after 4 days. Upon cessation of 3,3′,5′-Trit, the BMR returned in the next 20 days to line #4, 108.5 ml. O_2. A second course of 3,3′,5′-Trit gave similar results. After withdrawal of the daily thyroxine, the BMR returned to its starting level after 2 weeks.

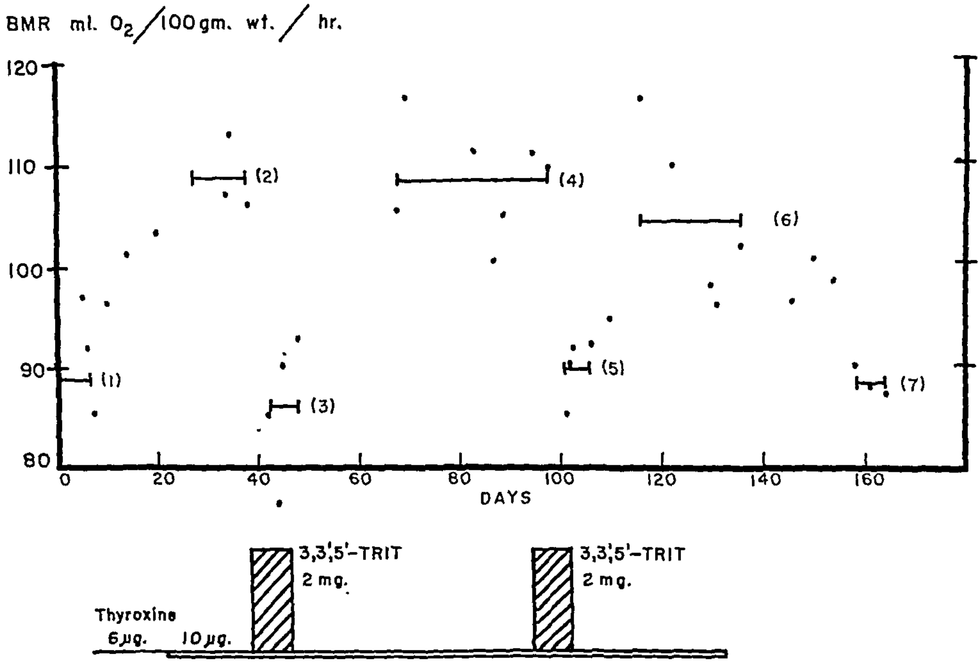

FIG. 1. Response of oxygen consumption of thyroidectomized rats to thyroxine and 3,3′,5′-triiodothyronine. The horizontal lines represent averages over the time periods indicated. The doses shown for thyroxine and 3,3′,5′-Trit are mg./kg./d.

Results from this group of animals indicate a 100% inhibition of thyroxine maintenance of metabolism at a molar ratio of 200:1 for DL-3,3′,5′-Trit:L-thyroxine. Another group of 2 animals studied through one course of 3,3′,5′-Trit responded with a 93% inhibition at a molar ratio of 100:1.

The effects of 3,3′,5′-Trit on the tissue metabolism from rats injected with a larger dose of thyroxine are shown in Figure 2. Each value represents the average of 5 tissues, liver, kidney, salivary glands, diaphragm, and heart from 2 or 3 animals. Using blank-injected rats as controls, injections of 0.25 mg. of thyroxine per kg. for 4 days gave a 22.9% increase in tissue oxygen consumption. When 10 mg./kg. of 3,3′,5′-Trit was injected simultaneously along with the thyroxine, the increase of tissue oxygen consumption was 18.7%, while the increase was only 7.4% in rats receiving 20 mg. 3,3′,5′-Trit per kg. in a similar fashion.

The results from tissue studies indicate a 9.6% inhibition of thyroxine effect at a

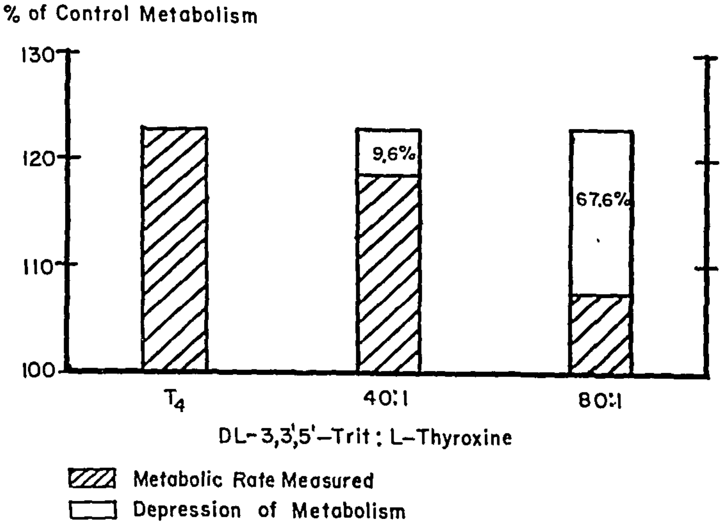

FIG. 2. Summated tissue oxygen consumption of hypothyroid rats treated with thyroxine alone or combined with 3,3',5'-triiodothyronine. Thyroxine dosage 0.25 mg. /kg./d., 3,3',5'-Trit at molar ratios of 40 or 80.

molar ratio of 40:1 for DL-3,3',5'-Trit: L-thyroxine and a 67.6% inhibition at a molar ratio of 80:1.

DISCUSSION

It is apparent that DL-3,3',5'-Trit in an amount 80 to 100 times that of L-thyroxine is able to interfere with the metabolic effects of the hormone. In all experiments the inhibitory effect was titrated against exogenous thyroxine in completely thyroidectomized animals. Consequently, the applicability of our findings to the therapy of euthyroid or hyperthyroid states seems highly questionable. Preliminary evaluation in athyreotic patients maintained on desiccated thyroid, thyroxine or Trit shows a considerable depression of BMR, but no effect in euthyroid subjects (personal communication from Dr. S. R. Hill).

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ANNOUNCEMENTS

ENDOCRINOLOGY STUDY SECTION

The Endocrinology Study Section, National Institutes of Health, announces that it is prepared to distribute, free of charge to qualified investigators, limited amounts of purified sheep pituitary follicle-stimulating hormone. The preparation has been packaged in two forms. 1) vials containing 5 mg. for use only as a Standard in assay procedures; 2) vials containing 25 mg., for experimental use. The preparation is a sterile, lyophilized powder, free or nearly free of other hormonal activities. Detailed specifications will be distributed with each sample.

Application for supplies, either of the Standard or of the experimental lots, should be made by letter to Dr. R. T. Hill, Executive Secretary, Endocrinology Study Section, National Institutes of Health, Bethesda 14, Maryland. The letter should state briefly an outline of the intended work and an estimate of the amount needed. Applications will be screened by a subcommittee and allotments will be made according to the estimates of merit of the intended work.

The preparation and distribution of FSH is supported by funds granted by several Institutes of the National Institutes of Health. The program is supervised by a subcommittee consisting of Drs. Warren O. Nelson, Roy W. Hertz, Robert W. Bates, R. T. Hill, and Alfred E. Wilhelmi, Chairman. The hormone preparation was made at Emory University under the immediate direction of Dr. Stanley Ellis.

1959 LAURENTIAN HORMONE CONFERENCE

The 1959 Laurentian Hormone Conference of the AAAS will be held at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period August 30–September 4. Investigators interested in attending this Conference should make application to the Committee on Arrangements of the Laurentian Hormone Conference, 222 Maple Avenue, Shrewsbury, Massachusetts, at an early date and in any event no later than May 11, 1959. A conference rate of $13.00 per day per person is extended to all invited participants. Since the number of our participants is necessarily limited by available accommodations, all applications are screened and invitations to attend are issued by the Committee on Arrangements by June 12. The following program has been arranged.

LAURENTIAN HORMONE CONFERENCE, INC.

Mont Tremblant Lodge

AUGUST 30–SEPT. 4, 1959

I. MECHANISM OF HORMONE ACTION

Participation of steroid hormones in the enzymatic transfer of hydrogen.
Paul Talalay and H. G. Williams-Ashman, The Ben May Laboratory, University of Chicago

Sunday evening, August 30

An enzymatic basis for the physiologic function of estrogens.
Claude A. Villee and Dwain D. Hagerman, Harvard University Medical School, Boston
Sunday evening, August 30

Effects of steroid hormones on some coenzyme mediated enzymatic reactions.
Lewis L. Engel and Jesse F. Scott, John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, and Departments of Biological Chemistry and Anatomy, Harvard Medical School
Monday morning, August 31

The mechanism of action of some estrogenic hormones on metabolism.
Kenneth W. McKerns, Edwin Kaleita and Paul H. Bell, Lederle Laboratories, American Cyanamid Company, Pearl River, New York
Monday morning, August 31

The role of cyclic adenylic acid in hormone action.
Robert C. Haynes Jr., E. W. Sutherland, Jr., and T. W. Rall, Western Reserve University School of Medicine, Cleveland
Monday morning, August 31

Cell permeability and hormone action.
O. Hechter and G. Lester, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts
Monday evening, August 31

II. Peptide Hormones

The immunological assay of human pituitary growth hormone.
Charles H. Read and George T. Bryan, State University of Iowa, University Hospital, Iowa City
Tuesday morning, September 1

Studies on erythropoetin.
W. F. White, Clifford W. Gurney and Eugene Goldwasser, Armour and Co. and Argonne Cancer Research Hospital, Chicago
Tuesday morning, September 1

Hormonal activity of active polypeptides.
Hector Croxatto and Livio Barnafi, Catholic University, Santiago, Chile
Tuesday evening, September 1

III. Thyroid Hormones

Studies on the chemistry and bioassay of thyrotropin from bovine pituitaries and transplantable pituitary tumors of mice.

Robert W. Bates and Peter G. Condliffe, National Institutes of Health, Bethesda

Wednesday morning, September 2

The transport of thyroid hormone and its relation to its metabolic fate.

Sidney Ingbar, Thorndike Memorial Laboratory, The Boston City Hospital, Boston

Wednesday morning, September 2

Re-evaluation of some concepts of iodine metabolism.

L. Van Middlesworth, The University of Tennessee, Memphis, Tennessee

Thursday morning, September 3

IV. HORMONES AND METABOLISM

Food and temperature.

John R. Brobeck, University of Pennsylvania School of Medicine, Philadelphia

Thursday morning, September 3

Influence of hormones on adipose tissue as a center of fat transport.

H. E. Wertheimer, The Hebrew University—Hadassah Medical School, Jerusalem, Israel

Thursday evening, September 3

Hormonal control of protein and fat metabolism in the pancreatectomized rat.

Robert O. Scow and Sidney S. Chernick, National Institutes of Health, Bethesda

Friday morning, September 4

Hormonal regulation of hepatic carbohydrate metabolism.

J. Ashmore, Indiana University Medical Center, Indianapolis, Indiana; George Cahill, Jr., New England Deaconess Hospital, Boston; and A. Baird Hastings, Scrips Metabolic Clinic La Jolla, California

Friday morning, September 4

THE AMERICAN GOITER ASSOCIATION

The 1959 meeting of the American Goiter Association will be held in the Drake Hotel, Chicago, Illinois, April 30, May 1 and 2. The program for the three day meeting will consist of papers and discussion dealing with the thyroid gland, its physiology, pharmacology, pathology and therapy.

Secretary
JOHN C. MCCLINTOCK, M.D.
149½ Washington Ave.
Albany 10, New York

STEROID BIOCHEMISTRY

The fourth session of the Training Program for Steroid Biochemistry sponsored by the U. S. Public Health Service will begin October 1. Applications are invited at the postdoctoral level for a one-year tenure with a stipend of $5500. The Program consists of lectures, planned laboratory, and eight months of research at the Worcester Foundation for Experimental Biology and Clark University or the University of Utah. Inquiries should be addressed to William R. Nes, Department of Chemistry, Clark University, Worcester, Massachusetts, or to Kristen Eik-Nes, College of Medicine, University of Utah, Salt Lake City. A predoctoral program also will be carried out in Worcester with stipends of $1800 for six months. All applications must be received prior to June 1.

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This Conference will afford the 57 speakers from all of Western Europe, Canada, Soviet Russia and the United States an opportunity to present their current work, discuss mutual problems and enlist suggestions, thereby preventing duplication of efforts.

NEW BOOK REGISTER

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ALDOSTERONE. An international symposium edited by Alex F. Muller and Cecilia M. O'Connor. In this symposium the biogenesis and its importance in clinical medicine are critically evaluated in light of our present knowledge. Boston: Little, Brown & Co. 232 pages. $8.00

THE LIPOPROTEINS METHODS AND CLINICAL SIGNIFICANCE. Edited by F. Homburger and P. Bernfield, Basel. New York: S. Karger. 94 pages. $4.00

DIE MEDIKAMENTÖSE BEHANDLUNG DER SCHILDDRÜSENERKRANKUNGEN. W. Grab and K. Oberdisse. Stuttgart: Georg Thieme Verlag. 285 pages; 55 illustrations. $9.00

KLINISCHE ENZYMOLOGIE. Rudolf Abderhalden. Stuttgart: George Thieme Verlag. 390 pages; 34 illustrations. $11.40

HORMONII STEROIZI. A. Lupulescu. Bucuresti: Editura Medicala. 400 pages; 146 illustrations.

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METABOLIC DISTURBANCES IN CLINICAL MEDICINE. Edited by G. A. Smart. Fourteen contributors. This book is designed to give definite help in diagnosis and treatment of metabolic diseases. Boston: Little, Brown & Co. Illustrated. 358 pages. $10.00

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THE BIOLOGY OF HAIR GROWTH. This book, written by twenty-one scientists, presents an up-to-date account of the organization, differentiation, and growth of hair. New York: Academic Press Inc. 520 pages. $15.00

L'ADRENALINE ET LA NORADRENALINE DANS LA REGULATION DES FONCTIONS HOMEOSTASIQUES. Colloques Nationaux de Centre National de la Recherche Scientifique. 250 pages.

THE CHEMICAL PREVENTION OF CARDIAC NECROSES. This book provides a complete synthesis of the current clinical and experimental observations on cardiac necroses. This information is viewed in the light of newly acquired knowledge about the production and prevention of the electrolyte-steroid-stress cardiopathies in animals. New York: The Ronald Press Co. 235 pages. $7.50

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CLINICAL OBSTETRICS AND GYNECOLOGY. Volume 1, No. 4. Symposium on Operative Obstetrics, J. Robert Willson, M. D., guest editor and Symposium on Genital Cancer, Daniel G. Morton, M. D., guest editor. New York: Paul B. Hoeber. $18.00 per year.

REPRODUCTIVE PHYSIOLOGY. Comparative reproductive physiology of domestic animals, laboratory animals and man. A. V. Nalbandov. San Francisco: W. H. Freeman & Co. 271 pages; illustrated. $6.75

CHLOROMYCETIN. Theodore E. Woodward and Charles L. Wisseman. Antibiotics monograph No. 8. New York: Medical Encyclopedia, Inc. 152 pages. $4.00

PENICILLIN. Harold L. Hirsh and Lawrence E. Putman. Antibiotics monogrpah No. 9. New York: Medical Encyclopedia, Inc. 148 pages. $4.00

STREPTOMYCIN AND DIHYDROSTREPTOMYCIN. Louis Weinstein and N. Joel Ehrenkranz. Antibiotics monographs No. 10. New York Medical Encyclopedia, Inc. 116 pages. $4.00

MODERN CHEMOTHERAPY OF TUBERCULOSIS. Roger S. Mitchell and J. Carroll Bell. Antibiotics monographs No. 11. New York: Medical Encyclopedia, Inc. 109 pages. $4.00

ENDOCRINOLOGY

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INHIBITION OF PITUITARY ACTH RELEASE: AN EXTRA-ADRENAL ACTION OF EXOGENOUS ACTH[1]

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ABSTRACT

Pituitary ACTH concentration is approximately doubled after administration of ACTH for one week to adrenalectomized rats, in confirmation of previous studies in intact animals. Attempts to determine the mechanism responsible for this extra-adrenal effect of exogenous ACTH yielded the following results: Pre-treatment with ACTH inhibits the pituitary ACTH depletion observed in adrenalectomized rats after application of a scalding stress. Similarly, ACTH pre-treatment blocks the fall in adrenal ascorbic acid obtained in intact control rats after the stress of ether anesthesia. The data are compatible with the hypothesis that the increase in pituitary ACTH concentration produced by exogenous ACTH is due to inhibition of endogenous ACTH release by the pituitary gland. The possibility that stimulation of ACTH synthesis or pituitary adsorption of exogenous ACTH may also occur after administration of ACTH cannot be excluded.

PREVIOUS studies (1) demonstrated that daily administration of ACTH gel to intact rats for one week resulted in a rise of 95% in pituitary ACTH concentration, whereas the administration of cortisone under identical conditions produced a 60% fall. Since the only commonly accepted effect of ACTH is stimulation of the adrenal cortex, ACTH administration might reasonably be expected to cause pituitary ACTH de-

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[1] This study was supported by Grants A-195 and RG-4315, National Institutes of Health, United States Public Health Service.

[2] Commonwealth Fund Fellow in Medicine.

[3] Fellow of the National Foundation.

pletion similar to that obtained with cortisone, rather than the increase in pituitary ACTH actually observed. The present study was undertaken to determine the mechanism of this unexpected action of injected ACTH.

METHODS

All experiments were performed in male rats of the Sherman strain, 90–140 gm., kept in a constant temperature room for at least one week prior to use. Diet consisted of Purina laboratory chow and water *ad libitum*. Control animals received physiological saline injected subcutaneously once daily. ACTH-treated animals received a long-acting ACTH preparation, either ACTH gel or Zinc ACTH, 4 or 6 U.S.P. units, injected subcutaneously once daily. Adrenalectomized rats were given their first injection immediately after operation and were maintained with supplementary 1% saline in the drinking water. Scalding served as a severe stress and consisted of immersion to the neck in water at 70° C. for 5 seconds under ether anesthesia. Ether anesthesia alone was used as a mild stress and was administered under the standardized conditions described by Wells *et al.* (2). Adrenal ascorbic acid levels were determined by the method of Mindlin and Butler (3).

The pituitary glands of the experimental animals were extracted for ACTH according to the method of Birmingham *et al.* (4). These extracts were subsequently assayed for ACTH either singly or in pools by incubation with rat adrenal slices *in vitro* by a modification of the technique of Saffran and Schally (5) previously described (1).[4] Individual pituitary extracts from ACTH gel-treated rats in the first experiment were assayed once each against randomly selected extracts from saline-treated rats. Pooled pituitary extracts in the second and third experiments were assayed 2 to 5 times each against their appropriate controls. Pituitary ACTH concentrations in the various experimental groups are expressed, therefore, as per cents of their respective control concentrations. The data were calculated in the form of concentration of ACTH per mg. anterior pituitary tissue; however, values for the total ACTH content of the individual pituitary glands or pools did not differ from those presented for concentration. Statistical calculations were performed according to the methods of Bliss (7). The values presented in the various tables are mean potency ratios with their approximate standard errors (Antilog $\overline{M} \pm 2.303\, s_{\overline{M}}$ Antilog $\overline{M}$). Statistical comparisons were based on the mean log potency ratios and their log standard errors ($\overline{M} \pm s_{\overline{M}}$). The number of replicate assays performed is noted in each table when indicated, as is the number of degrees of freedom (DF) for error available for statistical comparisons. The technique of bioassay of ACTH as employed permitted discrimination of differences in ACTH potencies as statistically significant when in excess of approximately 20%.

RESULTS

Experiment 1. The effect of ACTH pre-treatment on pituitary ACTH concentration in the absence of the adrenal glands was determined by administration of either saline (control) or 4 U.S.P. units of ACTH gel for 7 days to adrenalectomized rats. Both groups were sacrificed 24 hours after the last injection. A significant increase in pituitary ACTH concentration to 192% of the control level was observed in the rats given ACTH gel (Table 1). This rise is consistent with that previously obtained in intact rats (1).

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[4] Recently, Roberts (6) has questioned the specificity of the response of rat adrenal slices *in vitro* to ACTH. His conclusion of non-specificity was based on the observation that plasma obtained from hypophysectomized rats, as well as intact and adrenalectomized animals, stimulated corticosteroid release *in vitro*. Similar studies in this laboratory revealed that a small increment in corticosteroid release from adrenal slices occurred following the addition of plasma. However, this effect was constant and unrelated to the quantity of plasma added, i.e., a dose-response relationship could not be demonstrated. It should be noted that the amounts of plasma employed to produce such an increment represent quantities 2000–6000 fold greater by weight than the amounts of pituitary tissue actually used in the *in vitro* bioassay of ACTH.

TABLE 1. EFFECT OF EXOGENOUS ACTH ON PITUITARY ACTH CONCENTRATION IN ADRENALECTOMIZED RATS

Treatment	No. of rats	Pituitary ACTH as % control level*	P
Saline control	9	100	
ACTH gel	9	192 ±40	<.02

* Antilog $\overline{M} \pm 2.303\, s_{\bar{m}}$ (Antilog $\overline{M}$).

Experiment 2. Changes in pituitary ACTH concentration following ACTH administration in adrenalectomized rats were studied in greater detail after the injection of 4 U.S.P. units of ACTH gel daily for 8 days. Groups of 4 animals each were sacrificed immediately after the last injection (control) and at 2, 4, 6 and 24 hours thereafter. No change in pituitary ACTH concentration was obtained at 2, 4 or 6 hours after the last injection of ACTH in comparison to the control level (Table 2). A suggestive, but not statistically significant, decrease in pituitary ACTH was observed at the 2-hour interval. On the other hand, a significant increment above the control level was found 24 hours after the last injection of ACTH; this increase is equally significant when compared to the potencies obtained at the 2, 4 or 6 hour intervals. However, the possibility of a gradual accretion in pituitary ACTH prior to the sixth hour cannot be excluded entirely owing to the relative imprecision of the assay technique.

Experiment 3. The effects of pre-treatment with ACTH on pituitary ACTH concentration after the application of the severe stress of scalding were studied in 7 groups of adrenalectomized rats. Groups 1–3 were given saline daily and served as controls, whereas Groups 4 and 6 were given 4 U.S.P. units of Zinc ACTH daily and Groups 5 and 7 were given 6 U.S.P. units of ACTH gel daily. Each group was randomly divided into

TABLE 2. CHANGES IN PITUITARY ACTH CONCENTRATION IN UNSTRESSED ADRENALECTOMIZED RATS INJECTED WITH ACTH

Time after last ACTH injection (hours)	No. of rats	Pituitary ACTH as % control level*	P	No. of assays	DF
0	4	100			
2	4	81 ±15	n.s.	2	16
4	4	93 ±18	n.s.	2	16
6	4	102 ±12	n.s.	5	29
24	4	148 ±20	<.01	3	16

* Antilog $\overline{M} \pm 2.303\, s_{\bar{m}}$ (Antilog $\overline{M}$).

unstressed and stressed subgroups after 8 injections had been administered. The scalding stress was applied to Groups 1–5 two hours after the last injection and both the unstressed and stressed subgroups were sacrificed simultaneously 4 hours after stress. The same procedure was applied to Groups 6 and 7 twenty-four hours after the last injection of ACTH.

Scalding produced consistent and significant depletions in pituitary ACTH concentration in the saline-treated controls (Groups 1–3) to between 67 and 72% of the respective unstressed levels (Table 3) with a mean of 70%. On the other hand, application of the identical stress to rats 2 hours after the last injection of Zinc ACTH (Group 4) resulted in a significant rise in pituitary ACTH concentration to 122% of the unstressed level—an effect which differs strikingly from the stress-induced pituitary ACTH depletion observed in saline-treated animals ($P < .01$). Rats pretreated with ACTH gel (Group 5) and scalded 2 hours after the last injection showed a pituitary ACTH concentration of 88% of the unstressed level, a difference which is not statistically significant. The possibility remains that a relatively small fall in pituitary ACTH may have occurred within the limits of discrimination of the assay technique. However, the post-stress potency is significantly higher than that obtained after stress in the saline-treated controls ($P < .05$). Groups 6 and 7, stressed 24 hours after the last injection of Zinc ACTH or ACTH gel respectively, also showed no significant difference between unstressed and stressed pituitary ACTH concentrations. However, the post-stress values do not differ significantly from those obtained in the saline-treated controls (Groups 1–3).

TABLE 3. EFFECTS OF EXOGENOUS ACTH ON PITUITARY ACTH CONCENTRATION AFTER SCALDING STRESS IN ADRENALECTOMIZED RATS

Group	Treatment	No. of rats Stressed/Unstressed	Pituitary ACTH as % unstressed level*	P	No. of assays	DF
1	Saline	6/5	72 ± 10	< .05	3	36
2	Saline	6/5	72 ± 6	< .01	3	24
3	Saline	3/4	67 ± 10	< .01	4	20
	6 Hours After					
4	Zinc ACTH	6/5	122 ± 10	< .05	3	24
5	ACTH gel	3/4	88 ± 11	n.s.	4	31
	24 Hours After					
6	Zinc ACTH	3/3	80 ± 11	n.s.	3	24
7	ACTH gel	4/3	84 ± 20	n.s.	3	28

* Antilog $\overline{M} \pm 2.303\, s_{\overline{m}}$ (Antilog $\overline{M}$).

Experiment 4. The effects of ACTH pre-treatment on pituitary ACTH release after a mild stress was studied in intact rats after injection of either saline or 4 U.S.P. units of Zinc ACTH daily for 3 days. Twenty-four hours after the last injection, each group of animals was divided into unstressed and stressed subgroups and the latter subjected to ether anesthesia under standardized conditions. One hour later all the animals were sacrificed. The stress of ether anesthesia produced a significant fall of 30% in adrenal ascorbic acid in intact control animals given saline (Table 4). On the other hand, no change in adrenal ascorbic acid was observed after stress in rats pre-treated with Zinc ACTH. To determine whether adrenal responsiveness to ACTH remained intact after pre-treatment with Zinc ACTH, hypophysectomized rats were similarly pre-treated with Zinc ACTH for 3 days starting 24 hours after operation. Twenty-four hours after the last injection, the left adrenal gland was removed under Nembutal anesthesia and 4 milliunits of aqueous ACTH injected intravenously. One hour later the right adrenal gland was removed. A 31% depletion of adrenal ascorbic acid resulted after ACTH injection, suggesting normal adrenal responsiveness.

TABLE 4. EFFECT OF EXOGENOUS ACTH ON ADRENAL ASCORBIC ACID AFTER ETHER STRESS IN INTACT RATS

Treatment	Subgroup	No. of rats	Adrenal ascorbic acid (mg./100 gm.)*	P
Saline	Control	10	441 ± 26	< .001
	Stressed	10	310 ± 17	
Zinc ACTH	Control	9	410 ± 22	n.s.
	Stressed	10	413 ± 18	
Hypophysectomy *and* Zinc ACTH	Control (Left Adrenal)	5	321 ± 14	< .001
	ACTH (i.v.) (Right Adrenal)	5	221 ± 7	

* Mean ± Standard Error of the Mean.

DISCUSSION

The finding that ACTH administration produced a rise in pituitary ACTH concentration in adrenalectomized rats comparable to that observed previously in intact rats indicates that this effect of exogenous ACTH does not require mediation by the adrenal gland. A further increase in pituitary ACTH concentration was observed during the 24-hour period following the eighth daily injection of ACTH. The major portion of this rise occurred between the sixth and twenty-fourth hours with no apparent change in pituitary ACTH concentration prior to the sixth hour.

Three hypotheses may be invoked to explain these data. An increased blood level of ACTH may 1) result in non-specific ACTH adsorption by the pituitary gland; 2) stimulate ACTH synthesis; or 3) inhibit endogenous ACTH release with accumulation of normally synthesized hormone.

The last hypothesis was selected for further investigation. A scalding stress produced definite pituitary ACTH depletion in control animals.

Pre-treatment with ACTH modified this effect when stress was applied 2 hours after the last injection. An absolute rise rather than a fall in pituitary ACTH was observed after scalding in rats given Zinc ACTH. Pituitary ACTH concentration in rats pre-treated with ACTH gel after stress was higher than that observed in stressed control animals. Conclusions concerning these findings are subject to several considerations. Pituitary ACTH concentration at any moment is a measurement of a dynamic balance between the rate of ACTH synthesis and the rate of ACTH release; a change in concentration represents, therefore, a shift in this balance and not a direct measurement of either process alone. Thus, a fall (or negative balance) in pituitary ACTH concentration, such as that obtained after scalding in control animals, indicates that more ACTH was was released than synthesized from the time of stress to the time of sacrifice in response to the stressful stimulus. The degree of depletion measured is only a minimal estimate of the total quantity of ACTH actually released since newly synthesized ACTH may have been stored and/or released concurrently. On the other hand, pre-treatment with ACTH resulted in varying degrees of positive ACTH balance after scalding. Less ACTH was released than was synthesized and stored in stressed rats pre-treated with Zinc ACTH. The balance observed after stress in rats given ACTH gel was equivocal but was also more positive than that obtained in stressed control animals. Inhibition of pituitary ACTH release by exogenous ACTH with retention of newly synthesized hormone is suggested by these findings. Apparently, Zinc ACTH produced more complete inhibition of ACTH release than did ACTH gel. This observation perhaps may be explained by differences in the rates of absorption of the two preparations from sites of injection. Neither pituitary adsorption of exogenous ACTH nor accelerated synthesis of ACTH as a result of ACTH administration alone are applicable as explanations because 1) ACTH administration in the absence of stress did not increase pituitary ACTH concentration during the first 6 hours after the last injection—the period in which study of the effects of stress were completed; and 2) either ACTH adsorption or accelerated synthesis of ACTH, if present, should have affected the unstressed and stressed subgroups equally and are excluded, therefore, by the experimental design itself. An alternative explanation of the data would require the new hypothesis that ACTH pre-treatment resulted in acceleration of synthesis of ACTH (or increased pituitary adsorption of exogenous ACTH) only in the presence of stress but had no such effect in the absence of stress. Of course, this hypothesis does not explain the rise in pituitary ACTH obtained after ACTH pre-treatment in unstressed animals (Expts. 1 and 2). Significant inhibition of ACTH release as a result of pre-treatment with either preparation of ACTH was not demonstrated when the severe stress of scalding was applied 24 hours after the last injection.

A specific test of the inhibitory effect of pre-treatment with ACTH on pituitary ACTH release 24 hours after the last injection was performed with the milder stress of ether anesthesia. Since measurements of pituitary ACTH concentration before and after stress permit only indirect evaluation of the amount of ACTH released, alterations in adrenal ascorbic acid were employed as a more direct index of endogenous ACTH release. The data indicate that inhibition of pituitary ACTH release was present 24 hours after the last injection of Zinc ACTH and are in agreement with the previous findings. Since administration of various adrenal steroids may also result in inhibition of ACTH release (8), the effects of exogenous ACTH obtained in intact rats might conceivably be explained by the action of steroids secreted as a result of ACTH stimulation. However, the dose of corticosterone, the major corticoid hormone of rat adrenal glands, required to block ACTH release after stress apparently exceeds that amount maximally produced by the rat (9).

The administration of a long-acting ACTH preparation results in a significant rise in pituitary ACTH concentration in the adrenalectomized as well as the intact rat. The data presented support the hypothesis that this increase in ACTH concentration is due to inhibition of endogenous ACTH release from the pituitary gland. The findings neither support nor specifically refute the possibility that adsorption of exogenous ACTH or stimulation of ACTH synthesis may also occur. No conclusion may be made concerning the locus of this extra-adrenal action of ACTH, such as the pituitary gland itself or higher centers in the hypothalamus. Furthermore, since relatively large doses of ACTH were employed, these findings may well represent a pharmacologic rather than a physiologic effect of ACTH.

ADDENDUM

Since this paper was submitted for publication, Hodges and Vernikos (*Nature* 182: 725, 1958) have reported that less ACTH was discharged in response to ether stress as blood ACTH concentration increased following adrenalectomy in rats. These findings represent additional evidence in support of the hypothesis that pituitary ACTH release is regulated by the circulating level of ACTH.

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STUDIES ON THE CONTROL OF CORTICOTROPHIN RELEASE

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ABSTRACT

Different substances known to occur in hypothalamic nuclei and neurones were studied as ACTH releasers (adrenal ascorbic acid depletion method) in normal rats, in hypophysectomized rats, in hypophysectomized rats bearing a functional pituitary graft in the anterior chamber of the eye, and in rats in which the ACTH discharge following non-specific stimuli had been blocked by administration of 9-alpha-fluoro-hydrocortisone.

Adrenaline, noradrenaline, acetylcholine, histamine, serotonin, Pitressin, Pitocin, lysine-vasopressin and synthetic oxytocin were found highly effective in normal animals; Euler's substance P did not produce adrenal ascorbic acid depletion in normal rats.

Acetylcholine, histamine, serotonin, Pitocin, synthetic oxytocin and adrenaline were ineffective when tested in the hypophysectomized-grafted animals; only Pitressin gave a significant fall in adrenal ascorbic acid concentration when directly applied to the grafted pituitary gland.

In the steroid-blocked rats Pitressin, Pitocin and lysine-vasopressin were active as ACTH releasers when given through intravenous injections; the intracarotid injection of small doses of Pitressin was also active in the blocked rats; the other stressors were inhibited after steroid administration.

These results are discussed in connection with the hypothesis that posterior pituitary principles could play a role in the physiological regulation of ACTH output.

THE view that hypophyseal portal vessels regulate anterior pituitary function by transmitting a hypothalamic humoral substance, has received support from the results of several anatomical and physiological studies. In confirmation of earlier observations on amphibians and rats (1, 2) Worthington (3) has recently shown that blood in the portal vessels of the mouse flows from the capillaries in the median eminence to the sinusoids of the pars distalis; moreover Landsmeer (4), Jewell (5) and Daniel *et al.* (6) have recently demonstrated in dogs that the blood reaching the neurohypophysis via the posterior lobe artery, may readily flow into the anterior lobe through an additional system of anastomoses.

Several suggestions have been put forward as to the nature of the postulated hypothalamic neuro-humor: adrenergic substances (7), histamine

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(8), serotonin (9), substance P (10), lipoid or lipoprotein present in hypothalamic extracts (11), etc.

In recent years attention was drawn to a possible relationship between the antidiuretic hormone (ADH) of the posterior pituitary system and ACTH secretion. This hypothesis is supported by the following evidence: a) extracts containing the antidiuretic activity of the posterior lobe are effective in inducing ACTH release in normal animals (12, 13, 14, 15), in hypophysectomized animals bearing a functional pituitary graft in the anterior chamber of the eye (16), and in rats with hypothalamic lesions which block the response to non-specific stimuli (17); b) a defect in ACTH release is correlated with diabetes insipidus in rats with hypothalamic lesions (17); c) the neurosecretory material stored in the neurohypophysis and containing ADH may be depleted by noxious stimuli which also induce ACTH release (18, 19); d) ADH and ACTH are discharged simultaneously after the exposure to stressful stimuli (20) and after the administration of several drugs (21, 22).

It seemed of interest to re-investigate *in vivo* the effect of different substances, known to occur in hypothalamic nuclei and neurones (10, 23, 24, 25), on ACTH release, using normal rats, hypophysectomized rats, hypophysectomized rats bearing a functional pituitary graft in the anterior chamber of the eye, and rats in which the ACTH discharge following non-specific stimuli had been blocked by previous administration of adrenocortical steroids.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, weighing 150–200 gm., were used throughout these experiments. All animals were kept at a constant room temperature of 26–28° C.; they were given a 5% glucose solution to drink and maintained on a stock diet. The ACTH-releasing effect of adrenaline-HCl, noradrenaline-HCl, acetylcholine-HCl, histamine acid phosphate, serotonin creatine-sulphate, substance P,[2] commercial, purified and synthetic antidiuretic and oxytocic hormones (Pitressin, Pitocin, Syntocinon and lysine-vasopressin[3]) was studied in normal, hypophysectomized, hypophysectomized-grafted and corticoid-blocked rats. The ACTH-releasing effect of the different drugs was evaluated by means of adrenal ascorbic acid depletion (26).

Normal rats received the different drugs intraperitoneally, the control group being injected with NaCl 0.9% solution. The hypophysectomized rats were prepared by the parapharyngeal approach and used in the ascorbic acid depletion test 24 hours later. The hypophysectomized-grafted rats were prepared according to a procedure previously described (16) and used 1 month after grafting. The study of the modifications induced by pituitary transplantation on endocrine glands weight and morphology forms the subject of separate communications (16, 27); it is worth anticipating that the adrenal weight of the grafted animals was found lower than in intact controls, although much higher than in complete hypophysectomized animals. In the grafted rats the different drugs, dissolved in 0.1 cc. of saline, were directly injected into the anterior chamber of the graft-containing eye.

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[2] Two samples of substance P were obtained through the courtesy of Professor Gaddum (horse gut, 13.5 u./mg.) and of Professor Von Euler (cow gut, 100 u./mg.).

[3] Pitressin, Lot #31846 and Pitocin, Lot #209050 were kindly supplied by Dr. D. A. McGinty of Parke, Davis and Co.; Syntocinon was kindly supplied by Dr. A. Cerletti of Sandoz Ltd., Basle; lysine-vasopressin was obtained through the courtesy of Dr. G. A. Overbeek of N. V. Organon, Oss.

The hydrocortisone inhibited rat has been recently proposed for the study of hypophysiotropic substances, because of its failure to react with ascorbic acid depletion to unilateral adrenalectomy (28). Casentini *et al.* (29) reported that several other natural or synthetic steroid which exhibit adrenocortical activity block ACTH discharge after ether anesthesia and unilateral adrenalectomy. 9-alpha-fluoro-hydrocortisone proved to be 10 times as potent as hydrocortisone; this steroid was chosen for the experiments of the present paper. The steroid was administered intraperitoneally as a suspension in 0.3 cc. of an aqueous system of 0.9% NaCl; 0.4% Tween 80; 0.5% carboxymethylcellulose; 0.9% benzyl alcohol; the steroid inhibited rats were used in the ascorbic acid depletion test 4–6 hours after the injection of the blocking agent. In these rats, as well as in the hypophysectomized ones, the different drugs were usually given intravenously; in some experiments the blocked-rats received intracarotid injections. In hypophysectomized, in hypophysectomized-grafted and in steroid-blocked rats, right adrenalectomy was performed under ether anesthesia, to obtain a control level of ascorbic acid; the test-drug was administered immediately thereafter.

All animals were killed by decapitation 1.5 hours after treatment and the left adrenal was immediately removed. Adrenal glands were rapidly dissected free of fat and weighed on a torsion balance; their content of ascorbic acid was then estimated by the method of Roe *et al.* (30).

At the time of the autopsy the sella turcica of the hypophysectomized and hypophysectomized-grafted rats was examined with a magnifying glass for hypophyseal fragments, completeness of hypophysectomy being later confirmed by microscopic examination of serial sections through the sellar region.

The significance of the differences between the means obtained was calculated by Student's "t" test.

RESULTS

Normal rats

Confirming earlier observations the intraperitoneal injection of adrenaline (31), noradrenaline (32), acetylcholine (22), histamine (32), serotonin (9), Pitressin (13), Pitocin (13) was followed by a highly significant fall in adrenal ascorbic acid concentration ($P < 0.001$) in normal animals (Table 1); lysine-vasopressin and synthetic oxytocin also showed marked ACTH releasing activity in normal rats (Table 1). Two different samples of substance P were completely ineffective in the ascorbic acid depletion test (Table 1); this last result differs from Swingle *et al.* (33).

Hypophysectomized rats

Pitressin, Pitocin and lysine-vasopressin were tested in hypophysectomized rats in order to exclude the possibility of contamination with ACTH.

Table 2 shows that Pitressin, Pitocin and lysine-vasopressin, at the dose administered, do not induce significant ascorbic acid depletion in hypophysectomized rats.

TABLE 1. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN NORMAL RATS

Treatment	No. of rats	Adrenal ascorbic acid concentration (mg./100 gm. adrenal) Mean ±S.E.*	Ascorbic acid depletion from controls (mg./100 gm. adrenal)	P (Fisher's table)
NaCl 0.9% i.p.†	26	411± 8		
Adrenaline 0.05 mg.i.p.	6	304±46	−107	<0.001
Noradrenaline 0.05 mg.i.p.	11	307±14	−104	<0.001
Acetylcholine 0.20 mg.i.p.	10	331±13	− 80	<0.001
Histamine 0.20 mg.i.p.	18	345± 7	− 66	<0.001
Serotonin 0.05 mg.i.p.	6	299±42	−112	<0.001
Substance P (horse) 10 u.i.p.	6	427±43	+ 16	
Substance P (cow) 10 u.i.p.	6	436±28	+ 25	
Pitressin 0.3 u.i.p.	12	302±14	−109	<0.001
Pitocin 0.3 u.i.p.	23	358±12	− 53	<0.001
Lysine-Vasopressin 0.3 u.i.p.	18	311± 8	−100	<0.001
Synthetic oxytocin 0.3 u.i.p.	23	358±16	− 53	<0.005

* S.E. = Standard Error of the mean.
† i.p. = intraperitoneally.

TABLE 2. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY PITRESSIN, PITOCIN AND LYSINE-VASOPRESSIN IN HYPOPHYSECTOMIZED RATS

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ±S.E.*	P (Fisher's table)
Unilateral adrenalectomy plus			
NaCl 0.9% i.v.†		+56±12	
Pitressin 0.3 u. i.v.	6	+16±36	
Pitocin 0.3 u. i.v.	6	−35±17	>0.20
Lysine-vasopressin 0.3 u. i.v.	5	+11±12	

* S.E. = Standard Error of the mean.
† i.v. = intravenously.

Hypophysectomized-grafted rats

After severance of the pituitary stalk and transplantation of the pituitary to a distant site in the body, the rat no longer responds with adrenal ascorbic acid depletion to the stress of ether anesthesia and unilateral adrenalectomy (Table 3); in the grafted rats acetylcholine, histamine, serotonin, Pitocin and synthetic oxytocin were completely ineffective as ACTH-releasers, when injected into the anterior chamber of the graft-containing eye.

Adrenaline gave a positive response in one rat, a result which cannot be considered as significant.

Among the drugs tested only Pitressin gave a significant fall ($P < 0.005$) in adrenal ascorbic acid concentration, when directly applied to the grafted pituitary gland.

Steroid blocked rats

A. *Intravenous injections.* The sensitivity of the steroid inhibited rat to stressful stimuli and to ACTH administration has not been fully evaluated so far. To make certain that the site of the blocking action of the

TABLE 3. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN HYPOPHYSECTOMIZED-GRAFTED RATS: LOCAL ADMINISTRATION TO THE GRAFT-CONTAINING EYE

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ±S.E.*	P (Fisher's table)
Unilateral adrenalectomy plus			
NaCl 0.9%	6	+59 ±12	
Adrenaline 0.005 mg.	4	−27 ±29	N.S.
Acetylcholine 0.03 mg.	10	+ 6 ±11	
Histamine 0.05 mg.	5	+34 ±15	
Serotonin 0.005 mg.	5	+37 ±15	
Pitressin 0.01 U.	6	−56 ±11	<0.005
Pitocin 0.01 U.	6	−15 ±13	N.S.
Synthetic oxytocin 0.01 U.	6	− 1 ±20	

* S.E. = Standard Error of the mean.

steroid was not at the adrenal level, the adrenal ascorbic acid response of 9-alpha-fluoro-hydrocortisone blocked rats to different intravenous dosages of ACTH was examined.

Figure 1 shows that in blocked rats a linear log-dose relationship can be obtained with doses of ACTH ranging from 1 mU. to 16 mU. per rat; the ACTH-sensitivity of the steroid-blocked rats appears to be similar to that of normal animals but less than that of hypophysectomized animals (34).

When testing the different drugs in the 9-alpha-fluoro-hydrocortisone inhibited rats (Table 4) it became apparent that many stressors were completely ineffective as ACTH releasing agents. Only Pitressin, Pitocin and lysine-vasopressin could overcome the blockade, but only in the case of Pitressin and lysine-vasopressin the degree of significance was similar to that obtained in the normal animals.

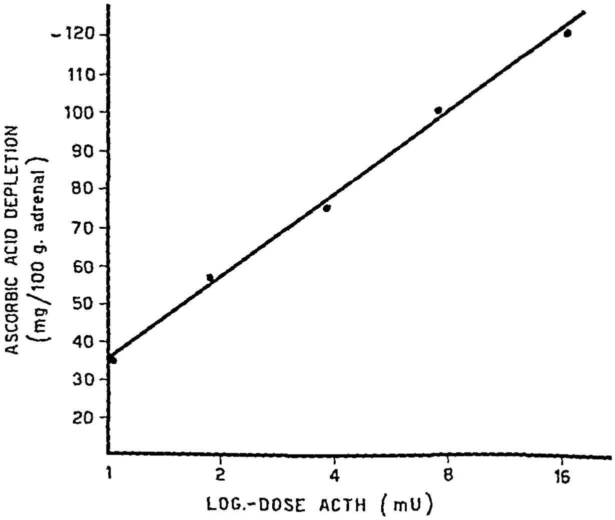

FIG. 1. Adrenal ascorbic acid depletion induced by intravenous injections of ACTH in 9-alpha-fluorohydrocortisone blocked rats.

TABLE 4. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN RATS BLOCKED BY 9-ALPHA-FLUOROHYDROCORTISONE (0.9 MG./100 G.B.W.)

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ±S.E.*	P (Fisher's table)
Unilateral adrenalectomy	6	+29 ±16	
Unilateral adrenalectomy plus			
NaCl 0.9% i.v.†	6	+18 ± 7	
Adrenaline 0.05 mg.i.v.	6	− 4 ±17	
Noradrenaline 0.05 mg.i.v.	6	+31 ±14	
Acetylcholine 0.20 mg.i.v.	12	−36 ±22	>0.1
Histamine 0.20 mg.i.v.	6	−25 ±15	>0.1
Serotonin 0.05 mg.i.v.	9	−27 ±19	>0.1
Substance P (horse) 10 U.i.v.	6	+28 ±15	
Substance P (cow) 10 U.i.v.	16	+19 ±23	
Pitressin 0.3 U.i.v.	19	−70 ±12	<0.001
Pitocin 0.3 U.i.v.	10	−41 ±17	<0.05
Lysine-vasopressin 0.3 U.i.v.	18	−78 ±11	<0.001
Synthetic oxytocin 0.3 U.i.v.	7	−27 ±29	>0.30

* S.E. = Standard Error of the mean.
† i.v. = intravenously.

B. *Intracarotid injections.* An objection against ADH as the possible physiological ACTH-stimulating substance was raised by the fact that rather high dosages of the hormone are usually required to elicit significant adrenal ascorbic acid depletions.

It seemed of interest to study whether the injection of Pitressin into the carotid blood, bringing the stimulating substance directly to the adenohypophysis, could allow a reduction of the amounts to be injected.

Table 5 shows that intracarotid injections of small doses of Pitressin are still active in the blocked rats; neither Pitocin nor synthetic oxytocin were active when given in small amounts by intracarotid injections in the blocked animals.

Figure 2 shows that Pitressin gives a linear log-dose relationship in doses ranging from 6 mU. (0.1 μg.) to 24 mU. (0.4 μg.) when given through intracarotid injections in 9-alpha-fluoro-hydrocortisone inhibited rats.

TABLE 5. ADRENAL ASCORBIC ACID DEPLETION PRODUCED IN 9-ALPHA-FLUORO-HYDROCORTISONE BLOCKED RATS BY INTRACAROTID INJECTIONS OF PITRESSIN, PITOCIN AND SYNTHETIC OXYTOCIN

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ±S.E.*	P (Fisher's table)
Unilateral adrenalectomy plus			
NaCl 0.9% i.c.†	10	− 3 ±19	
Pitressin 0.012 U.i.c.	9	−87 ±30	<0.025
Pitocin 0.012 U.i.c.	4	+16 ±26	
Synthetic oxytocin 0.012 U.i.c.	5	−15 ±38	>0.20

* S.E. = Standard Error of the mean.
† i.c. = intracarotid injection.

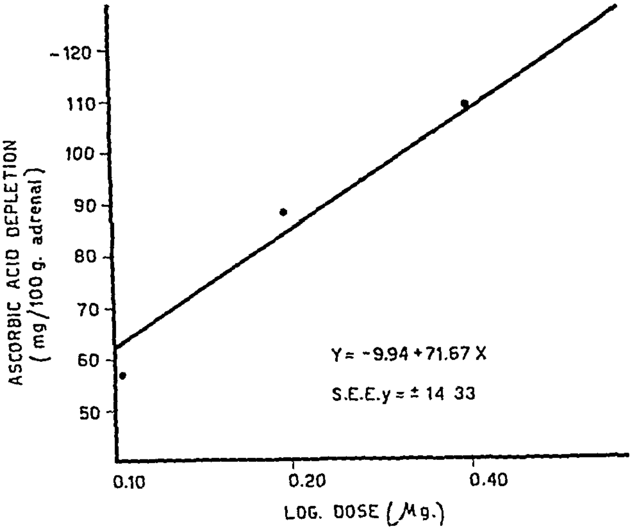

FIG. 2. ACTH releasing activity of intracarotid injections of Pitressin in 9-alpha-fluorohydrocortisone blocked rats. Pitressin = 60 U./mg.

DISCUSSION

Cheng *et al.* (35), McDermott *et al.* (36), Fortier (37) had shown in rats that adrenaline and histamine may induce ACTH release by the transplanted pituitary; similarly Hume (38) and Fortier *et al.* (39) observed in stalk-sectioned dogs and rabbits normal eosinopenic responses to adrenaline. In contradiction with these results our own observations seem to indicate that adrenaline and histamine (as well as acetylcholine and serotonin) induce ACTH release not through a direct action on hypophyseal glandular cells but probably through a nervous mechanism. This hypothesis is supported by many recent observations: Hume (40), Porter (41) and McCann (42) reported that hypothalamic lesions prevent the eosinopenia and the adrenal ascorbic acid depletion induced by adrenaline, histamine and Pitocin; Olling *et al.* (43), Wells *et al.* (44), Sevy *et al.* (45) and Van Peenen *et al.* (46) observed that certain nervous depressants (morphine, reserpine, chlorpromazine, etc.) inhibit the hypersecretion of ACTH in response to histamine, adrenaline and noradrenaline; Porter (41) and Nakayama (47) found electrical activation of hypothalamic nuclei after giving adrenaline and histamine; Schally *et al.* (48) and Gullemin *et al.* (49) found histamine, noradrenaline and synthetic oxytocin inactive in stimulating ACTH release from pituitaries incubated *in vitro*. An hypothalamic site of action for adrenaline and acetylcholine is also suggested by the studies on the mechanism of the antidiuretic action of these drugs (21, 22).

The ability of Pitressin to discharge ACTH when applied to the graft-containing eye is in agreement with an earlier observation (16) and shows that Pitressin directly acts on hypophyseal glandular cells; a similar conclusion was reached by McCann (42) working in hypothalamic-lesioned animals, and by Sevy *et al.* (45) working in chlorpromazine-inhibited rats. Sayers' observation (50) that sections through the brain stem at the upper level of the pons abolish the pituitary response to Pitressin is contradicted by the ACTH releasing activity shown by Pitressin when directly injected into the grafted pituitary.

The ability of steroids with adrenocortical activity to suppress the release of ACTH after unilateral adrenalectomy is in agreement with the results and conclusions of various workers (see 29 for references). Some recent results seem to suggest that adrenocortical steroids block ACTH secretion by acting upon nervous structures: in agreement with this hypothesis Courrier *et al.* (51) reported that labeled cortisone is concentrated *in vivo* by hypothalamic and posterior pituitary tissues; on the other hand McCann (42) Fulford *et al.* (52) and Ganong *et al.* (53) reported that the adrenal ascorbic acid response to unilateral adrenalectomy as well as the compensatory adrenal hypertrophy which normally follows unilateral adrenalectomy are suppressed by hypothalamic or stalk lesions. In contradiction to these results Fortier *et al.* (39) recently reported that stalk section in rabbits exerts little effect on the adrenal ascorbic acid depletion following unilateral adrenalectomy.

The experiments presented here provide two pieces of evidence of the fact that adrenal steroids normally act at some site proximal to the pituitary: 1) in the hypophysectomized grafted animals unilateral adrenalectomy was not followed by adrenal ascorbic acid fall in the remaining adrenal; 2) in agreement with previous observations (42, 54, 55, 56) Pitressin (the only agent which exhibited a direct stimulating activity on hypophyseal glandular cells when tested in the grafted animals) was found able to overcome the steroid blockade.

The results obtained injecting Pitressin into the carotid artery of corticoid-blocked rats seem to indicate that the pituitary is very sensitive to this stimulus; they also rule out the possibility of a direct action of Pitressin at the level of the adrenal cortex (57).

The ACTH-sensitivity of the 9-alpha-fluorohydrocortisone treated normal rats was found similar to that of untreated normal controls; this result, as well as Hodges' observations (58) in the DCA-treated normal rats, seems to rule out the possibility of a block of ACTH activity at the adrenal level; two recent reports (34, 59) deal with the reduced ACTH sensitivity of hypophysectomized corticoid-treated animals.

A few results had already shown that the corticoid-blocked rats no longer respond to histamine (54, 55, 60) and adrenaline (56, 61, 62). The results here presented extend these investigations and show that in the 9-alpha-fluoro-hydrocortisone inhibited rats the ACTH releasing activity of noradrenaline, acetylcholine and serotonin is also blocked; this last result was recently confirmed (56).

The 9-alpha-fluoro-hydrocortisone blocked rats, because of their specific response to posterior pituitary preparations (Pitressin, lysine-vasopressin and Pitocin), proved to be very useful for testing the ACTH releasing activity of hypophysiotrophic hypothalamic substances; the steroid blocked rats could also be employed in substitution of hypophysectomized animals in the biological assay of ACTH.

Our experiments though suggesting that a neurohumoral substance which stimulates the release of ACTH by acting directly at the hypophysial level is present in posthypophysial extracts do not identify it specifically; in particular they do not exclude the possibility that the effect observed after the administration of Pitressin and purified lysine-vasopressin could be due not to ADH itself but to a contaminant with specific ACTH releasing properties (CRF) as suggested by Saffran's (63) and Guillemin's *in vitro* experiments (49);[4] it should however be pointed out that it has recently been demonstrated that synthetic lysine-vasopressin also has an ACTH stimulating activity both *in vitro* and *in vivo* (65, 66, 67).

Evidence is now accumulating showing that posterior pituitary principles may play a role in the release of other pituitary trophic hormones, such as TSH (68, 69), STH (70), gonadotrophic hormones (71) and prolactin (72).

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[4] Some doubts have been recently raised on the specificity of these *in vitro* methods (64).

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THE ADRENOTROPIC ACTION OF HUMAN PREGNANCY PLASMA[1]

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ABSTRACT

Jailer and coworkers have reported that the plasma of pregnant women contained an adrenotropin which was distinct from ACTH; they showed that treatment of freshly hypophysectomized rats with pregnancy plasma partially prevented adrenal involution. We have found evidence suggesting that the adrenotropic action of pregnancy plasma is attributable to its content of chorionic gonadotropin. The gonadotropin appears not to act directly on the rat adrenal; instead it stimulates the interstitial cells of the testis, and their steroid secretory product in turn acts on the adrenal to produce a partial maintenance of adrenal weight.

JAILER and coworkers (1) have reported that plasma from pregnant women and from patients with Cushing's disease associated with adrenal hyperplasia contained an adrenotropic factor which was distinct from ACTH. This material partially prevented adrenal weight loss in hypophysectomized rats, but did not cause ascorbic acid depletion. We wondered whether the adrenotropic action of pregnancy plasma might be related to its content of chorionic gonadotropin (CG) and have made studies with this in mind. Our findings lead us to believe that CG in pregnancy plasma is responsible for the partial weight maintenance of the adrenal glands in hypophysectomized rats. However, CG does not act directly on the adrenal gland. It stimulates the interstitial cells of the testis in the hypophysectomized rats and the steroid secretory product of these cells then acts on the adrenals to produce a partial weight maintenance.

METHODS

Adrenotropic action was tested in individually caged male Sprague-Dawley rats hypophysectomized on the 40th day of life. Animals were obtained from the Hormone Bioassay Laboratories, Chicago, except where noted. Hypophysectomized female rats, intact male rats, and hypophysectomized, gonadectomized male rats were used as indicated in certain experiments. Injections of test material were begun on the 41st day and given subcutaneously in a volume of 0.3 cc. three times daily for 8 days (once daily for testosterone propionate in sesame oil). The rats were then killed by ether anesthesia and the two adrenals dissected and weighed together. In some cases testicular and adrenal tissues were fixed in 10% formalin for histological section. There were 6–10 indi-

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vidually caged animals in each treatment group. In these and in the other animal experiments mentioned subsequently, animals were assigned to treatment groups by random numbers, and the data were collected without knowledge of the treatment involved.

Chorionic gonadotropin was assayed by the rat ovarian hyperemia method as described by Albert and Berkson (2). One cc. of test material was injected subcutaneously into 30 to 50 gm. intact female rats; plasma samples were diluted at least tenfold with saline because of the inhibiting effect of higher concentrations of plasma in this test. Four hours after injection the animals were killed by ether anesthesia and the ovaries inspected. A dose response curve to standard chorionic gonadotropin in normal saline was plotted on semi-log paper. Our dose response curve was not identical with that obtained by Albert and Berkson, but the response was linear over the range of 16 to 85% positive responses (2–8 I.U. CG). The linear portion of the curve was plotted using data from 110 animals in 10 experiments. Unknown materials were diluted so that the response to one or more dilutions fell within the linear range. A minimum of 5 animals were tested at each dosage for both unknown and standard materials.

ACTH activity was measured by adrenal ascorbic acid depletion in hypophysectomized rats as described by Sayers and coworkers (3), using an Armour preparation with potency stated in international units as a standard. Five animals per group were used, each animal serving as its own control.

Blood from pregnant women was collected in heparin usually within 15 minutes of delivery, iced immediately, and centrifuged in a refrigerated centrifuge. The supernatant plasma was removed and frozen. Pools of plasma were prepared by combining individual specimens.

Chorionic gonadotropin, (Ayerst) was supplied as a dry powder prepared from human pregnancy urine. The stated potency was 1225 I.U./mg. We have used this material as our standard.

RESULTS

On the basis of the ovarian hyperemia assay, 4 maternal plasma pools contained from 22 to 58 I.U./cc. of chorionic gonadotropin and all partially prevented adrenal weight loss in hypophysectomized rats (Table 1A). When maternal plasma pools were diluted, the minimum effective dose preventing complete adrenal weight loss contained between 0.5 and 2.7 I.U./cc. CG. Two samples of pooled human plasma obtained from the blook bank contained no measurable CG activity (sensitivity limit 2 I.U./cc). and had no effect on the rat adrenal weights. However, the addition of chorionic gonadotropin to bank plasma in amounts between 11 and 165 I.U./cc. produced the same effect on the adrenals as did the undiluted maternal plasma pools. Smaller amounts of CG were then added to the bank plasma to determine the minimum effective dose, which was found to contain between 0.5 and 1 I.U./cc. CG. As may be seen from Table 1A, similar amounts of CG occurring naturally in maternal plasma or added to bank plasma produced similar effects on adrenal weight.

For purposes of comparison, gonadotropic substances from other sources were tested both for CG activity and prevention of adrenal weight loss. Sheep pituitary luteinizing hormone (LH) (Armour) containing 1 mg. Armour standard per cc. was found to be equivalent to 8 I.U./cc. CG in the ovarian hyperemia assay. This material was effective in preventing adrenal

TABLE 1

Material	CG≈ I.U./cc.	Adrenal weight (mg)	
		Mean	Std. error of mean
A. *The relationship of adrenal weights in hypophysectomized rats to the dosage of chorionic gonadotropin (CG) or its equivalent as measured in the rat ovarian hyperemia assay*			
Controls			
Saline	0	15.1	0.6
Non-injected	0	14.8	1.3
Maternal plasma pool			
1	58	21.8	0.7
2	46	20.7	1.2
3	25	21.3	0.5
4	22	21.0[a]	1.0
1 (1:10 dilution)	5.8	17.3	0.7
2 (1:10 dilution)	4.6	16.1	0.5
5 (1:10 dilution)	2.7	18.9	0.6
5 (1:50 dilution)	0.5	16.3	0.4
Bank plasma pool			
1	0*	14.9[b]	0.6
2	0*	15.9[c]	1.0
Chorionic gonadotropin added to bank plasma	165	20.0	0.6
	17	20.6	0.5
	11	22.0	0.8
	11	20.8	0.7
	3.3	19.1[d]	0.6
	1	19.2	0.6
	0.5	16.0	1.0
	0.05	14.2	0.6
Luteinizing hormone	8	20.8	1.0
Equine gonadotropin	7	19.7	1.0
B. *The relationship of adrenal weights in hypophysectomized rats to the dosage of ACTH*			
	ACTH I.U./cc.		
ACTH	0.28	21.8	0.6
	0.083	19.4[e]	0.5
	0.028	17.0	0.6
Bank plasma Pool 2	0	15.9[f]	1.0

[a–b, c–d, e–f] Test for significant difference by analysis of variance: $P < 0.01$ in each of these pairs.

* Sensitivity limit: 2 I.U./cc.

weight loss to the same extent as a corresponding dose of CG. Similarly, equine gonadotropin (Ayerst) in concentration of 150 I.U./cc. was equivalent to 7 I.U./cc. CG in the ovarian hyperemia assay and was also effective in preventing adrenal weight loss.

ACTH produced no ovarian hyperemia in the assay for CG, but, as expected, it was effective in preventing adrenal weight loss in hypophysectomized rats (Table 1B).

Histological sections of adrenal from hypophysectomized rats showed that maternal plasma (Fig. 3), CG (Fig. 5), LH, and ACTH (Fig. 7) produced a wider cortex than that seen in untreated animals (Fig. 1). The results of these various treatments were indistinguishable in hematoxylin and eosin stained sections. In Sudan stained sections, however, only ACTH treatment (Fig. 13) was associated with a fairly uniform distribution of fat-staining material, and disappearance of the sudanophobic zone (cf. Fig. 10, 11, 12 and 14). Histological sections from the testes likewise revealed a difference between the effect of ACTH (Fig. 8) and that of the other agents. Maternal plasma (Fig. 4), CG (Fig. 6) and LH maintained the interstitial cells of the testes, whereas ACTH did not.

Injections of bank plasma in control animals resulted in a thinning of the adrenal cortex with atrophy and hypoplasia most pronounced in its central portion. A sudan-free zone in the fascicular zone was present; the interstitial cells of the testes were not maintained.

Neither maternal plasma nor CG contained ACTH activity as measured by adrenal ascorbic acid depletion (Table 2). Equine gonadotropin and sheep pituitary LH produced slight depletions in adrenal ascorbic acid; the effect of each was less than that produced by 0.0011 I.U. ACTH.

Neither CG nor maternal plasma prevented adrenal involution in either hypophysectomized *female* rats or in hypophysectomized *and gonadectomized* male rats (Table 3A and B). However, partial adrenal weight maintenance could be effected in hypophysectomized gonadectomized male rats by the sole administration of testosterone propionate (Table 3C). These rats were of the same strain but were obtained from a different source. The degree of weight maintenance and the histological effects on the adrenal were identical with those previously obtained with CG and maternal plasma in hypophysectomized male rats with intact testes.

CG did not affect adrenal weights in intact male rats (Table 3D).

DISCUSSION

These observations lead us to believe that the adrenotropic action of pregnancy plasma was caused by CG, which stimulated the testes of the test animals to produce a steroid secretory product. The steroid in turn

TABLE 2. ASSAY FOR ACTH ACTIVITY*

Material	Dose/rat	Ascorbic acid (mg/100 gms. adrenal tissue)		
		Before ACTH	After ACTH	Net change
ACTH	0.011 I.U.	496	271	−225
ACTH	0.0011 I.U.	475	367	−108
Sheep luteinizing hormone	2 mg Armour standard	496	441	− 55
Equine gonadotropin	600 I.U.	479	460	− 19
Chorionic gonadotropin	1000 I.U.	414	442	+ 28
Maternal plasma pool 4	0.6 cc.	401	439	+ 38

[*] None of the materials tested had as much as 0.0011 i.u. ACTH activity.

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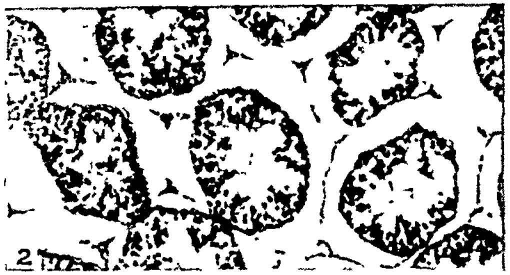
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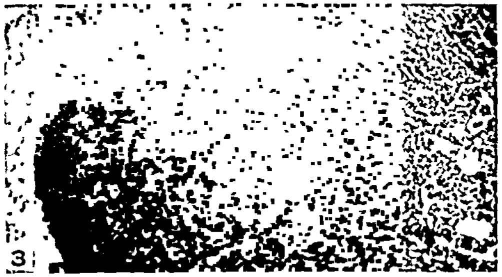
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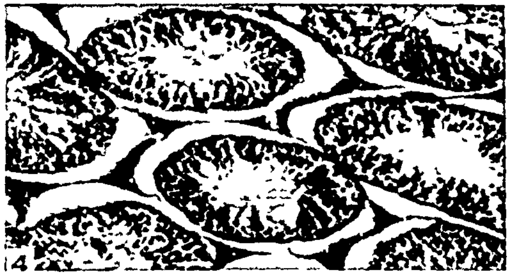
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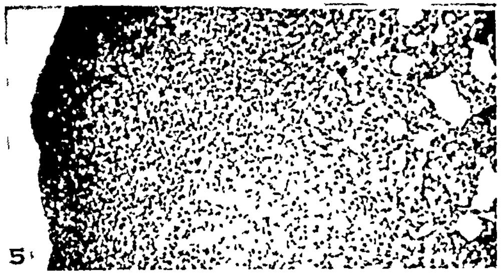
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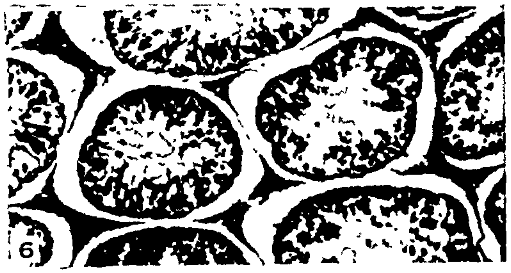
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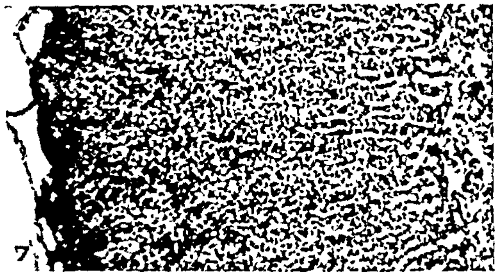
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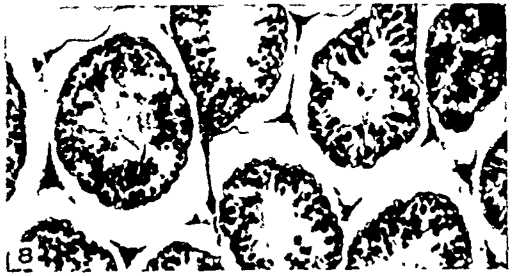
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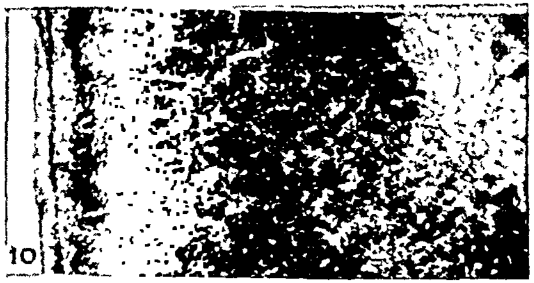
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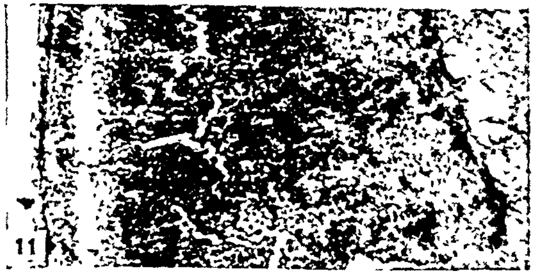
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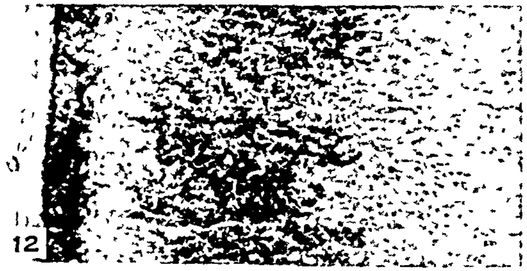
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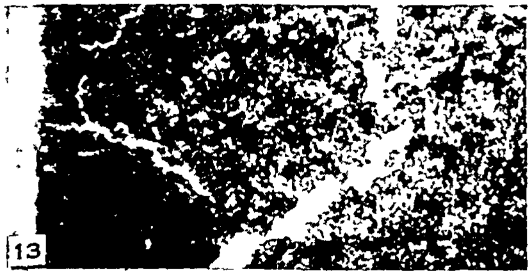
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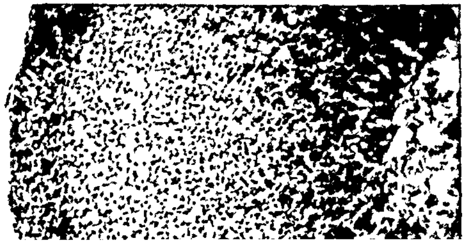

acted on the adrenals of the hypophysectomized rats to partially maintain their weight. Three types of studies are presented to afford evidence of this mechanism.

First, the adrenotropic action of pregnancy plasma was imitiated by CG and other gonadotropins with luteinizing hormone activity. Blood bank plasma pools which contained no CG activity had no adrenotropic action. When CG was added to the inert bank plasma in amounts comparable to those in pregnancy plasma, the preparation reproduced the adrenotropic action of maternal plasma. The minimum dose of each necessary to produce the adrenotropic response was the same in terms of CG activity, and the maximimum response to higher doses of each was the same. Both produced identical histological effects in the rat adrenals and maintained the interstitial cells of the rat testes. The adrenotropic and histological effects of maternal plasma and of bank plasma with added CG were reproduced by sheep pituitary LH and by equine gonadotropin. This parallelism

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FIGS. 1, 3, 5, 7, 9 adrenals stained with H&E (×35). Figs. 2, 4, 6, 8 testes stained with H&E (×35). Figs. 10–14 adrenals stained with Sudan III (×35).

FIG. 1. Adrenal from a hypophysectomized rat which received no therapy. The cortex is thin.

FIG. 2. Testis from a hypophysectomized rat which received no therapy. The interstitial cells are atrophic.

FIG. 3. Adrenal from a hypophysectomized rat treated with maternal plasma. The cortex is thicker than in the untreated animal.

FIG. 4. Testis from a hypophysectomized rat treated with maternal plasma. The interstitial cells are not atrophic.

FIG. 5. Adrenal from a hypophysectomized rat treated with chorionic gonadotropin. The cortex is thicker than in the untreated animal.

FIG. 6. Testis from a hypophysectomized rat treated with chorionic gonadotropin. The interstitial cells are not atrophic.

FIG. 7. Adrenal from a hypophysectomized rat treated with ACTH. The cortex is thicker than in the untreated animal.

FIG. 8. Testis from a hypophysectomized rat treated with ACTH. The interstitial cells are atrophic.

FIG. 9. Adrenal from a hypophysectomized caste rat treated with testosterone propionate. The cortex is thicker than in the untreated animal and resembles those seen after treatment with maternal palsma, chorionic gonadotropin, and ACTH.

FIG. 10. Adrenal from a hypophysectomized rat which received no therapy. A zone devoid of fat-staining material is present in the fascicular zone.

FIG. 11. Adrenal from a hypophysectomized rat which received maternal plasma. A zone devoid of fat-staining material is present in the fascicular zone.

FIG. 12. Adrenal from a hypophysectomized rat which received chorionic gonadotropin. A zone devoid of fat-staining material is present in the fascicular zone.

FIG. 13. Adrenal from a hypophysectomized rat which received ACTH. There is a fairly uniform distribution of fat in the cortex and no fat-free zone is present.

FIG. 14. Adrenal from a hypophysectomized, castrate rat which received testosterone propionate. A zone devoid of fat-staining material is present in the fascicular zone.

TABLE 3

	Adrenal weight (mg.)	
	Mean	Std. error of mean
A. *Female hypophysectomized rats*		
Control (Bank Plasma Pool 2)	17.1	0.3
Chorionic Gonadotropin (25 I.U./cc.)	17.6	0.8
Maternal Plasma Pool 5	18.8	0.9
B. *Male castrate hypophysectomized rats*		
Control (Bank Plasma Pool 2)	19.1	0.6
Chorionic Gonadotropin (25 I.U./cc.)	18.3	1.0
Maternal Plasma Pool 5	19.0	0.5
C. *Male castrate hypophysectomized rats**		
Control (sesame oil)	12.3	0.6
Testosterone propionate 2 mg./day	20.5	1.6
D. *Intact male rats**		
Control	42.4	1.4
CG (25 I.U./cc.)	40.0	0.9

A and B: Neither chorionic gonadotropin (CG) nor maternal plasma caused a change in adrenal weight in hypophysectomized female rats or in hypophysectomized castrate male rats. Control values in these animals are higher than in male non-castrate hypophysectomized rats and reflect higher adrenal weights at the beginning of treatment.

C: Increase in adrenal weight in hypophysectomized, gonadectomized male rats given testosterone propionate in sesame oil ($P < 0.01$).

D: CG had no effect on adrenal weights in intact male rats.

Analysis of variance revealed no significant differences within groups A, B, and D.

* From Charles River Breeding Laboratories, Brookline, Massachusetts.

of action between maternal plasma, CG, LH, and equine gonadotropin suggested that the active principle was luteinizing hormone activity, a property they shared in common as evidenced by the ovarian hyperemia response to each.

Second, ACTH also maintained adrenal weight in hypophysectomized rats, but ACTH activity in the various test materials cannot account for their effectiveness in adrenal weight maintenance. ACTH assay revealed that in the test doses employed, neither maternal plasma, CG, LH, nor equine gonadotropin contained sufficient ACTH for adrenal weight maintenance. For example 2 mg. of LH contained less than 0.0011 I.U. ACTH activity. The LH given in the adrenal weight maintenance experiments contained 1.0 mg./cc., or less than 0.00055 I.U./cc. ACTH activity. This was <1/150 the dose of ACTH required to produce an equivalent response (0.083 I.U./cc.; table 1B). Similar calculations revealed factors of <1/45 for maternal plasma, <1/300 for equine gonadotropin, and <1/25,000 for CG. The factors for maternal plasma and CG are conservative estimates; 0.0011 I.U. ACTH activity over-estimates their possible ACTH content, since neither showed any activity at all in the assay. In addition, histological evidence indicated the lack of ACTH activity in the test materials. In the absence of ACTH, a characteristic sudan free zone appears in the outer part of the fascicular zone (4) (Fig. 10). Adrenotropic doses of maternal plasma, CG, LH, and testosterone propionate failed to eliminate this zone in the hypophysectomized rats, but a similarly adrenotropic dose of ACTH caused it to disappear.

Third, maternal plasma and the gonadotropins which imitated its action all maintained the interstitial cells of the testis, suggesting that the secretory product of these cells might be the adrenotropin. Further experiments showed that this was the case. The presence of the testis was essential for either maternal plasma or CG to show an adrenotropic action. Neither of these materials prevented adrenal involution in either hypophysectomized female rats or hypophysectomized and gonadectomized male rats. However, testosterone propionate administered to hypophysectomized and gonadectomized male rats was effective in maintaining adrenal weight in the same way as CG or maternal plasma in hypophysectomized male rats. This effect of testosterone on the adrenal of hypophysectomized rats is confirmed by previous work of other investigators (5, 6, 7) whose studies demonstrated partial weight maintenance of the adrenal in hypophysectomized male rats, using doses of 1.25 to 2.5 mg. testosterone propionate. Our own experience and that of others (7) indicates that lower doses are less effective and that the effect disappears when the dose of testosterone is reduced to about 0.2 to 0.5 mg. per day.

Maintenance of adrenal weight by steroid therapy has also been reported in rats with adrenal atrophy induced by cortisone administration instead of by hypophysectomy (8, 9, 10). In these animals, a large number of C-19 and methylated C-20 steroids were shown to be effective to varying degrees in the prevention of adrenal weight loss. The most effective steroids were androgenic, although their effectiveness bore no constant relationship to androgenicity, chemical structure, or other recognized factor. These findings appear to support our interpretation of our own results in hypophysectomized rats, in which we believe that adrenal weight was partially maintained by steroids secreted by the rat testes under gonadotropic-stimulation.

We first suspected that CG might cause the partial adrenal weight maintenance in hypophysectomized rats because of previous evidence that CG was related to the striking adrenal hypertrophy in the human fetus (11, 12). This evidence remains attractive, but the mechanism by which we believe CG partially maintains the adrenal of hypophysectomized male rats does not appear to be directly applicable to the problem of human fetal adrenal hypertrophy for several reasons. First, the differences in adrenal weight which we have demonstrated represent only a partial prevention of involution. The adrenal weight of 40-day old intact male rats was 37.5 ±1.8 mg. At the end of the 8-day experiment, adrenal weights were of the order of 15 mg. for controls and 20 mg. for CG treated rats. Extensive involution had therefore occurred in both groups and had been only partially prevented by CG therapy. The human fetal adrenal, on the other hand, is greatly enlarged, being proportionately 10 to 20 times heavier than the adult gland (13). Second, CG had no effect on adrenal weights in non-hypophysectomized rats. In the human fetus the pituitary appears to be essential for the adrenal hypertrophy to occur; anencephalic infants, which have severely defective pituitaries, usually do not show fetal adrenal enlargement. Third, chorionic gonadotropin is found in the fetus only in low titers relative to those in the mother (14), and we have found (unpublished observations) that neither fetal urine nor fetal plasma is adrenotropic in the hypophysectomized male rat. We believe, therefore, that if chorionic gonadotropin is related to human fetal adrenal development, as is suggested by other experiments, factors in addition to those demonstrated in these studies must be involved.

Acknowledgments

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CHANGES IN THE GOLGI APPARATUS OF THE ISLETS OF LANGERHANS IN THE RAT FOLLOWING GLUCOSE AND INSULIN ADMINISTRATION[1]

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ABSTRACT

The appearance of the Golgi apparatus of the beta cells of the islets of Langerhans of the rat was studied with a view to finding a better histological criterion of activity than those now available. The Golgi apparatus of the beta cell is clearly distinguishable from that of the alpha cell, since the former is much larger and more elaborate, while the latter is very small and compact. Maintenance of elevated blood glucose levels for 4 hours resulted in an increase in the size and intricacy of the beta cell Golgi apparatus, while at 12 hours the change was even more marked. Attention was then turned to the alpha cells to see if maintenance of hypoglycemia, said to be a stimulus to glucagon release, would produce alterations of their Golgi bodies. Insulin was infused continuously intravenously, resulting in blood glucose levels of 15–30 mg.% over at least a 4-hour period. In spite of the marked hypoglycemia, no changes in the Golgi apparatus of the alpha cells was observed, although the beta cell Golgi apparatus showed beginning involution.

A GROWING body of evidence indicates that the blood glucose level is one of the most important factors influencing the rate of insulin production. This relationship was first demonstrated by Banting and Gairns (1) and Houssay, *et al.* (2), while in a more extensive study, Zunz and LaBarre (3), using cross-circulation experiments, showed that production of hyperglycemia in the donor dog resulted in a decline in the blood glucose level of the recipient. Local infusions of glucose into the pancreas have been demonstrated by Kosaka (4) and Brown *et al.* (5) to result in hypoglycemia; the latter authors have also observed islet hyperplasia, degranulation and beginning hydropic degeneration in the part of the pancreas infused with glucose. The most direct piece of evidence was provided by Anderson and Long (6), who found that elevation of the glucose level in the blood perfusing the isolated rat pancreas resulted in an increase in the insulin content of the blood.

Like the cells composing most glandular tissue, the beta cells of the

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islets of Langerhans show alterations in their morphology under conditions which may be expected to increase their activity. Prolonged hyperglycemia in cats and dogs (7, 8, 9, 10) results in degranulation and hydropic degeneration, while the usual response in the guinea pig (11, 12, 13) and the rat (14, 15) consists only of degranulation which is followed by islet hyperplasia if the elevation in blood glucose is sufficiently prolonged.

While the beta cells usually respond acutely to a stimulus by degranulating, nevertheless Nerenberg (16) has shown that this is not always the case, since, in his experiments, fasting reduced the granule content and feeding of carbohydrate restored it. Therefore, the granule content is an unreliable criterion of activity in these cells. Indeed, as has been clearly pointed out many times, the degree of granulation at any time depends upon the balance between production and release. Since there are no good criteria of acute changes in beta cell activity, the first group of experiments outlined below was undertaken in an effort to discover other more reliable morphological changes which might be used to assess the degree of beta cell activity and which would occur sufficiently rapidly to be useful in acute studies. The Golgi apparatus seemed to be a likely candidate for such a role. While the argument concerning the actual nature of the Golgi apparatus is still in progress, it is nevertheless generally recognized that, when glandular tissue shows increased secretory activity, the Golgi apparatus in each cell becomes larger and more elaborate.

Studies of the Golgi apparatus in the islets of Langerhans have been curiously few. Ludford and Cramer (17) described hypertrophy of the Golgi bodies in the islets during pregnancy, elevation of body temperature, and after operative procedures. Vazquez-Lopez (18), although unable to impregnate the Golgi apparatus in the normal islets, described what he termed an hypertrophied Golgi apparatus in the islets of estrogen-treated rats. In neither stuy was any differentiation made between the Golgi apparatus of alpha and beta cells.

While it is quite clear that the beta cells produce insulin and that an elevation in blood glucose leads to an increase in their activity, the situation with respect to glucagon is not as well worked out. Until recently, there has been controversy concerning the cell of origin of this hormone. Recently, however, deDuve (19) reported that complete destruction of the alpha cells by high doses of cobalt is associated with the absence of extractable glucagon. In addition, Bencosme and Liepa (20) and Bencosme, Liepa and Lazarus (21) have found that the uncinate process of the dog pancreas, a region almost devoid of alpha cells, is also almost devoid of glucagon. In view of these findings, the conclusion that the alpha cells produce glucagon is almost inescapable. However, the factors leading to its release are less well defined. Foa *et al.* (22), using cross-circulation experiments in which the pancreaticoduodenal vein of the donor dog drained into the portal circulation of the recipient, found that hypoglycemia in the donor dog resulted in hyperglycemia in the recipient, a finding which suggests that hypoglycemia is at least one of the ways by which glucagon release may be caused. It would therefore be anticipated that a prolonged depression of blood sugar level would result in morphological changes in the alpha cells due to their sudden increase in activity, and accordingly the second group of experiments was carried out as described below.

METHODS

Rats of the Long-Evans strain, 60–90 days of age and weighing 200–300 gm., were employed, female rats being used when glucose was infused and male rates when insulin was infused. All animals were fed a stock diet *ad libitum*. In some rats, diabetes was produced by the intravenous injection of 50 mg. per kg. of body weight of recrystallized alloxan following a fast of 72 hours. For the next 6 days, these animals were treated with 4 units per day of crystalline zinc insulin subcutaneously. At least 30 days then elapsed before the animals were used.

In the first group of experiments, glucose was infused continuously through a polyvinyl catheter in the tail vein. In the experiments of 4 hours duration or less, the animals were anesthetized with nembutal throughout, but in the 8 and 12 hour studies, the animals were anesthetized with ether during the placing of the catheter in the tail vein, and then allowed to recover. These animals were kept in restraining cages for the duration of the experiment. In the shorter experiments, the animals were first given a priming dose of 1 cc. of 25% glucose in 0.2% pyrogen-free NaCl intravenously, following which they were connected to a constant injection pump which delivered 5.3 cc. of 25% glucose in 0.2% NaCl per hour. In the 8 and 12 hour experiments, no priming dose was given and the glucose was infused as a 10% solution in 0.5% NaCl at a rate of around 15 cc. per hour. NaCl was included in the infusion to avoid depletion of body sodium and chloride as a result of the marked diuresis. At the end of each experiment, the infusion was stopped, the animals were anesthetized, and after a lapse of several minutes, blood was drawn from the vena cava for the measurement of blood sugar and the pancreas was removed and fixed for histological study.

In the second group of experiments, insulin was infused into both normal and alloxan-diabetic animals. Both groups of animals were infused with crystalline zinc insulin intravenously at a rate of 0.44 unit per hour (Lilly "Isletin" diluted with 0.9 pyrogen-free NaCl), but in addition, the alloxan-diabetic animals were given 2–3 units of insulin intraperitoneally two hours before the beginning of the experiment in order to bring the blood sugar levels down to roughly normal levels. As in the short glucose infusions, the animals were under continuous nembutal anesthesia from the time that the infusions were begun. Blood was drawn from the inferior vena cava every half or three-quarters of an hour for the determination of glucose. At the end of the experiment, the pancreas was removed and fixed.

Glucose was measured by the Nelson modification of the Somogyi method (23). Part of the pancreas was fixed in Bouin's fluid, embedded in paraffin, sectioned at 4 μ and stained with the Gomori aldehyde fuchsin following preliminary oxidation with 0.3% $KMnO_4$ in 0.3% H_2SO_4 for 1 minute with subsequent bleaching in 3% sodium bisulfite. A brilliant stain of the beta cell granules with almost no background staining was obtained in 4 minutes. After rinsing in 95% alcohol, the slides were stained for about 5 minutes in chrome hematoxylin (24), differentiated briefly in 1% HCl in 70% alcohol, washed in tap water for 5 minutes and counterstained for 20 minutes in the following mixture: 0.25 gm. chromotrope 2R, 0.1 gm. light green, 0.7 gm. phosphotungstic acid, 20 cc. glycerol, 80 cc. water, 1 cc. glacial acetic acid. Slides were rinsed briefly in 0.1%

acetic acid, dehydrated and mounted. The alpha cell granules were deep orange, the beta cell granules purple and the cytoplasm of both cells green. For demonstration of the Golgi apparatus, a second part of the pancreas was cut into small pieces and fixed for 24 hours in Champy-Kull fixative, washed in running water for 24 hours, then treated with 2% osmic acid at 40° C for 12 hours and at 35° C for 8 days. The tissues were washed for 24 hours in running water, dehydrated, embedded in paraffin, and sectioned at 4 μ. Sections too heavily impregnated with osmic acid were bleached in 0.125% $KMnO_4$ followed by 3% sodium bisulfite. Some preparations of pancreas impregnated with osmic acid were counterstained with Mallory-Azan for the differentiation of cell types.

RESULTS

The Normal Islet

The appearance of the islets of Langerhans in the normal rat following application of specific granule stains has been amply described. In all normal controls, the usual preponderance of beta cells over alpha cells was observed together with a general grouping of beta cells centrally and alpha cells around the periphery. While the beta cells of smaller islets were usually filled with granules, the largest islets contained numerous cells with granules confined to the borders of the cells.

The appearance of the islets after impregnation of the Golgi apparatus requires more extensive comment, inasmuch as so little work appears where this technique was used. When islets of normal animals were examined (Fig. 1), it was apparent at once that there were two types of Golgi apparatus and it was necessary to determine whether the cells possessing the two different Golgi types could be identified with the alpha and beta cells. Most of the cells in the islet had a relatively large, moderately elaborate Golgi body, which was located close to the nucleus and formed a sort of cap over it. The cells containing this type of Golgi apparatus were fairly large and formed the central part of the islet, thus correspondong in position and size, at least, to the beta cells. In addition, their cytoplasm contained granular material which had a tendency to reduce osmic acid, thus lending a greyish tint to the cytoplasm. Located at the edges of the islets, frequently in a cap at one end, were smaller cells, all of which had a clear cytoplasm and which had a very small, exceedingly simple Golgi apparatus near one pole of the nucleus. These cells, then, corresponded in their position and size to the alpha cells. Since it proved impossible to counterstain these preparations with either aldehyde fuchsin or chrome hematoxylin, they were finally counterstained with Mallory-Azan, with the result that the granules of the small cells with the small Golgi bodies stained with azocarmine, while the others did not, thus indicating that probably the former were the alpha cells. In an effort to put this distinction on a still firmer footing, the appearance of the islets of alloxan-diabetic rats was studied following impregnation of the Golgi apparatus (Fig. 2). It is well-known that rats severely diabetic as a result of alloxan treatment show almost complete disappearance of the beta cells, leaving islets composed almost exclusively of alpha cells. The few beta cells left are

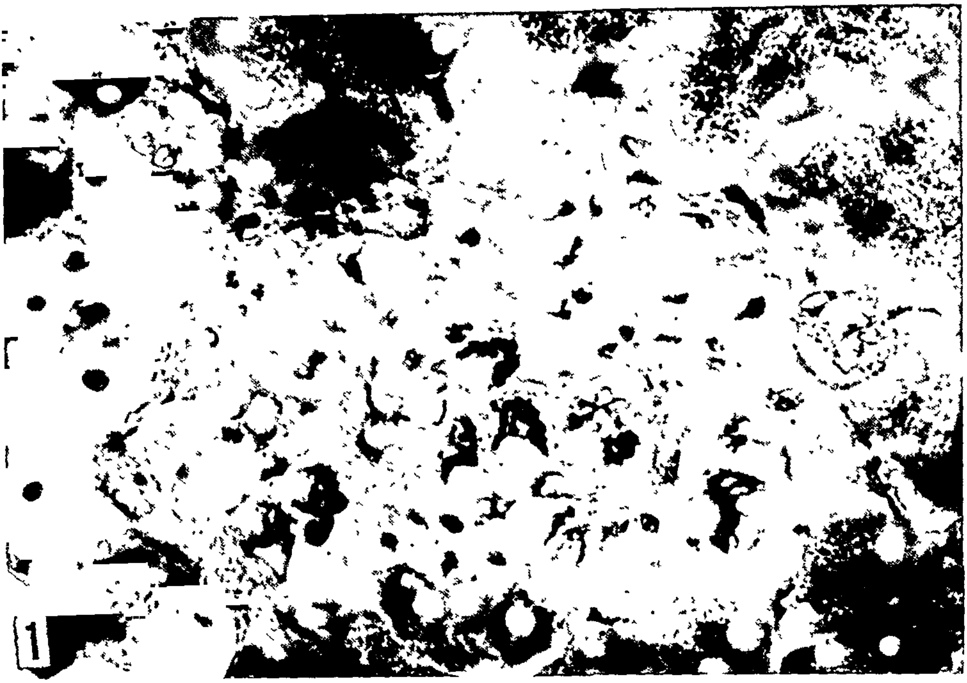

FIG. 1. Golgi preparation of islet from a normal rat. Note centrally placed large cells with large Golgi bodies and dark cytoplasm and peripherally placed small cells with small, very simple Golgi bodies. ×917.

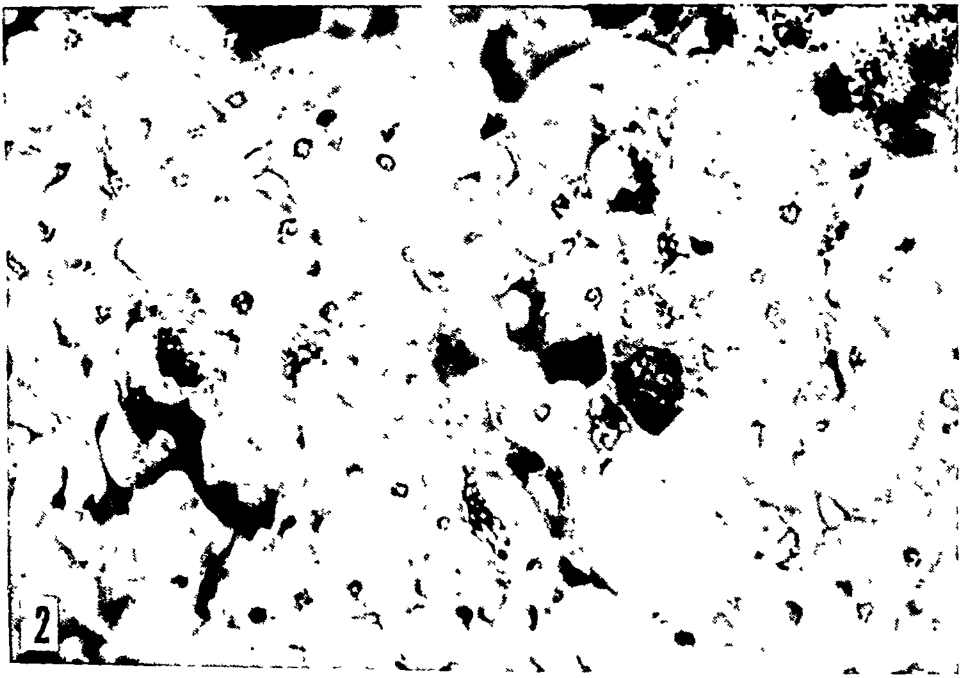

FIG. 2. Golgi preparation of islet from an untreated alloxan-diabetic rat. Note almost complete disappearance of beta cells, leaving the islet composed almost entirely of alpha cells with small Golgi bodies and clear cytoplasm. ×917.

usually considerably enlarged. In this series, these changes were confirmed in material fixed in Bouin's fluid and stained with aldehyde fuchsin. In the Golgi preparations from these animals, almost the entire islet was composed of the small cells with clear cytoplasm and small Golgi bodies. Occasionally, there were seen one or two quite large cells with greenish cytoplasm and an enormously large and elaborate Golgi apparatus. The latter cells would logically seem to be representatives of the few beta cells left, which had greatly hypertrophied as a result of the hyperglycemia, while the small cells appeared to be identical with the alpha cells.

Group I: Glucose Infusion

Sixteen experiments were carried out in which the duration of the infusion varied from 1½ to 12 hours. The infused animals were found to have blood sugar levels ranging from 327 to 1310 mg.% at the end of the experiments.

Following the infusion of glucose, the beta cell granulation was greatly reduced, a finding which has been repeatedly described by others. This degranulation was almost completely confined to the medium- and large-sized islets, with the small islets appearing not to participate in the response. In all experiments the degree of degranulation was about the same regardless of the duration of the hyperglycemia. The granulation of the alpha cells appeared to be unchanged.

In osmic acid preparations from these animals, the Golgi bodies of the beta cells had become somewhat larger and considerably more elaborate (Figs. 3, 4). This process was discernable in the animals infused for only 1½ hours, was well-established at 4 hours and was quite marked at 12 hours. The Golgi apparatus of the alpha cells did not appear to change in any way.

Group II: Insulin Infusion

Four normal animals were infused with insulin intravenously at a rate of 1.3–1.8 units kg./hr. The blood sugar declined promptly, reaching 40 mg.% or below within an hour, and was maintained at this level for an interval which varied from 1½–3 hours. This persistent hypoglycemia produced no change in the granule content or other appearance of either alpha or beta cells in the material stained with aldehyde fuchsin. There was a slight shrinkage of the Golgi bodies of the beta cells, but in the alpha cells they were unchanged.

Inasmuch as the number of alpha cells compared to beta cells was small in the normal animal, making their study more difficult, 5 alloxan-diabetic rats were infused with insulin in the same way and at the same rate as the normal rats, except that 2 nours before the beginning of the experiment, they were given 3 units of regular insulin to bring their blood sugars down into the normal range. For at least 4 hours, their blood sugars were between

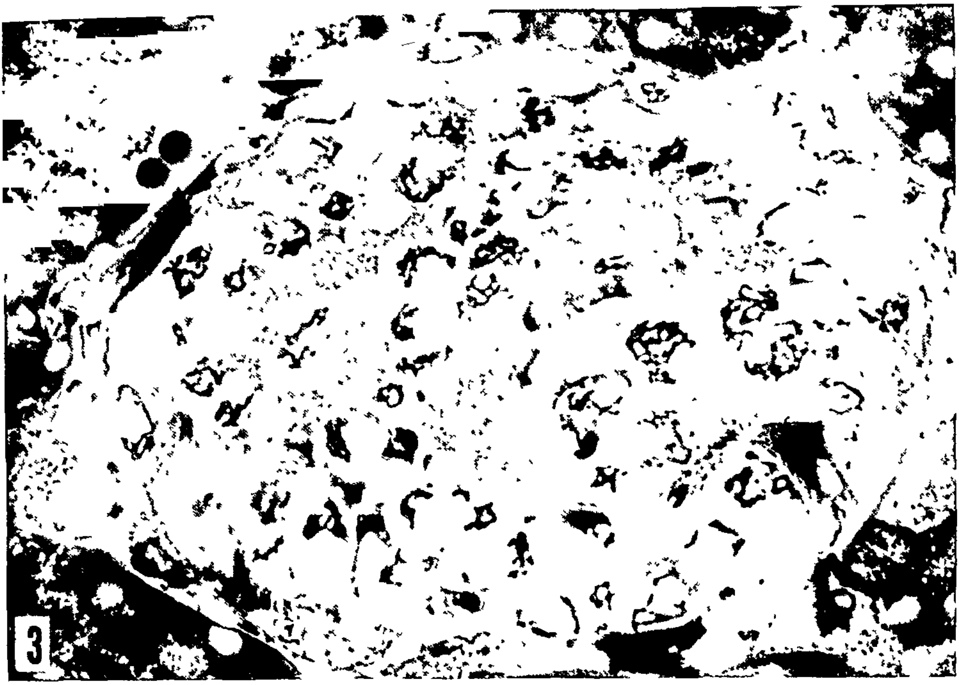

FIG. 3. Golgi preparation of islet from a normal control rat. Islet is composed almost entirely of beta cells. A few cells show elaborate Golgi bodies, but in general they are small and simple. ×917.

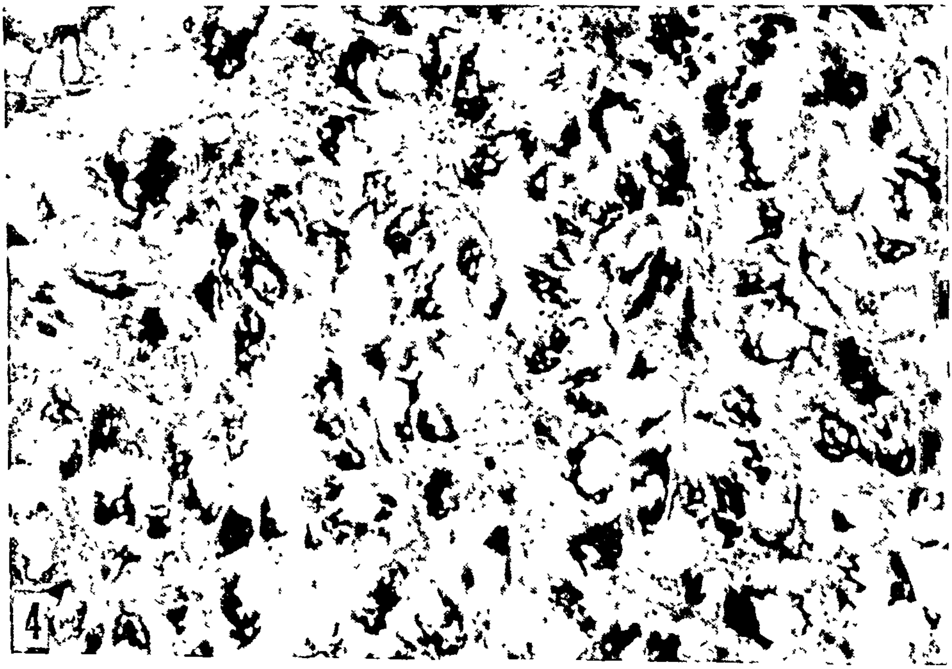

FIG. 4. Golgi preparation of islet from a normal rat infused with glucose for 4 hours. Note generalized increase in complexity of Golgi bodies in all cells. ×917.

15 and 30 mg.%. With the aldehyde fuchsin stain, the untreated alloxan-diabetic controls showed the usual masses of well-granulated alpha cells with an occasional degranulated beta cell. The animals infused with insulin showed no changes in the granule content of the alpha cells, nor did the few beta cells change in appearance. The appearance of the islets of untreated diabetic rats in Golgi preparations has been described above (Fig. 2). In the Golgi preparations from diabetic rats given insulin, none of the alpha cells showed any enlargement or elaboration of the Golgi apparatus (Fig. 5).

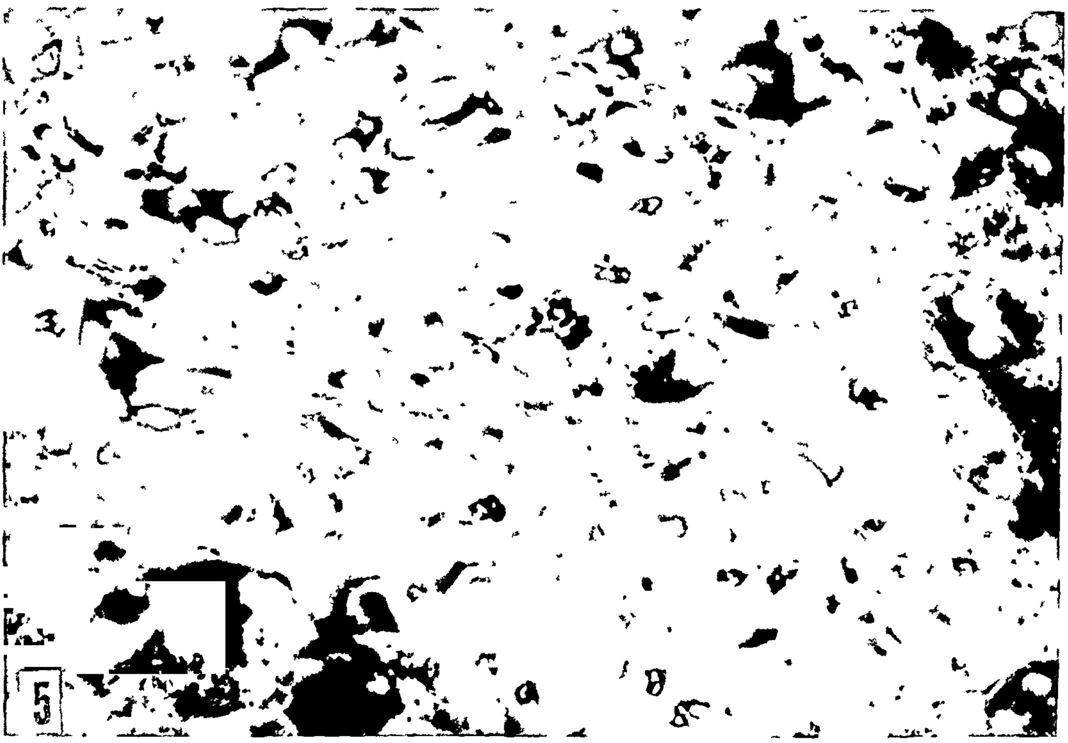

Fig. 5. Golgi preparation of islet from an alloxan-diabetic rat infused with insulin for 4 hours. Islet is composed almost entirely of alpha cells whose Golgi bodies are indistinguishable from those in Fig. 2. ×917.

DISCUSSION

Since the appearance of the Golgi apparatus changes fairly rapidly when the beta cells are presented with a stimulus to insulin production in the form of hyperglycemia, we have at hand a means of assessing the level of activity of these cells which is probably better than that afforded by observation of granule content alone, inasmuch as the amount of granular material undoubtedly represents a balance between production and release. If, as has been shown here, the Golgi apparatus of the beta cells changes its appearance within 4 hours after the beginning of an adequate stimulus to these cells, one might perhaps expect that the Golgi apparatus of the alpha cells would show recognizable alterations within roughly the same time interval following the beginning of hypoglycemia, which should be an adequate stimulus to their activity. The finding that the alpha cells did not change in any way is puzzling. Of course, several assumptions were made in this experiment. The first was that hypoglycemia is in fact a stimulus to the production of glucagon in the rat, as it has been reported to be in the dog (22). A second assumption was that glucagon is rapidly released when an adequate stimulus is provided. However, the available evidence does indicate that glucagon is released within minutes after the production of hypoglycemia (22). A third assumption was that the morphology of the alpha cells would change when they are stimulated in about the same length of time required by the beta cells to change. A fourth assumption is that the alpha cells are indeed the site of glucagon production, and, if it were not for the strong evidence already cited, one would be tempted to doubt that the alpha cells produce glucagon, since, in this experiment and in many others, their morphology remained unchanged under a variety of conditions said to produce release of glucagon. For example, Fodden and Read (25) reported increased blood levels of glucagon after 12 days of GH treatment, and Bornstein, Reid and Young (26) found a hyperglycemic effect of pancreatic venous blood of cats treated with GH for 5 days and in rats treated for 24 hours. Also Foa *et al.* (27) reported that single injections of GH resulted in the release of a substance from the pancreas which caused hyperglycemia in cross-circulation experiments. In spite of these findings, the reports of the effects both of hypophysectomy and of GH administration on the morphology of the alpha cells have been variable. Although most workers have found *no changes* after hypophysectomy, Ferner and Tonutti reported atrophy of the alpha cells (28). The reports of Ferner have been criticized by Volk, Goldner and Frank-Crowley (29) because of the capricious nature of the Gros-Schultze silver method used to demonstrate the alpha cells. The latter authors, using more reliable techniques, were unable to find any differences after hypophysectomy. The effect of GH administration has also been highly variable. The alpha cells have been reported as unchanged after GH treatment (30), while on the other hand others have observed increased mitotic activity (31), or some increase in size, but only when corticotropin was simultaneously administered (32). The effect of the other known stimulus for glucagon release, namely hypoglycemia, has not been investigated in chronic experiments with the exception of one study (33) in which the alpha:beta cell ratio was reported as increased following chronic insulin administration, but unfortunately, no consideration was given to the possibility that this might have been due to a decrease in number of the beta cells. If the alpha cells do produce glucagon under the variety of conditions just outlined, then they are remarkable in their ability to do so with a minimum of morphological change.

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A METHOD FOR THE BIOLOGICAL ASSAY OF PHOSPHATURIC ACTIVITY IN PARATHYROID EXTRACTS[1]

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ABSTRACT

A biological assay method has been developed for estimation of the phosphaturic activity of parathyroid extracts. It is based on the increased urinary excretion of inorganic phosphate by 2-month old male rats during the first 6 hours after parathyroidectomy. The extract to be tested is injected immediately after parathyroidectomy and just before beginning the collection of urine. Various factors of importance in the assay method were studied, including the use of parathyroidectomized rather than intact rats, the optimal time and the completeness of the urine collection, a stock diet rather than a low-calcium diet, and the dose-response curve. The most favorable conditions yielded a reasonably rapid and sensitive assay method with a mean index of precision (λ) of 0.30. Several applications of the method are described, in particular the estimation of the ratio of phosphaturic to calcium-mobilizing activity in parathyroid extracts.

A METHOD of biological assay for phosphaturic activity, for use in conjunction with a method (1, 2) already available for calcium-mobilizing activity, was needed to aid in the purification of parathyroid extract and to help settle the question whether a single hormone or two separate hormones are responsible for the two activities. This paper comprises a report of the experiments leading up to the development of such a bioassay method and descriptions of the recommended procedure and some of its applications.

MATERIALS AND METHODS

Male albino rats of uniform age obtained from the Holtzman Rat Co. were kept in pairs in suspended wire mesh cages in an air conditioned animal room with a controlled temperature of approximately 78° F. The stock diet was Purina laboratory chow. Analysis of a sample showed 0.94% calcium and 0.35% phosphorus. The low-calcium diet, a modification of the diet of Shaw (3), is described in Table 1. Parathyroidectomy was carried out under ether anesthesia by cauterization with a hot wire.[2] For collection of urine each rat was placed in an individual metal metabolism cage with drinking water available but no food. The spontaneously voided urine was collected in glass jars con-

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[2] Details of the technique of parathyroidectomy will be supplied on request.

TABLE 1. LOW CALCIUM DIET

	gm./kg.
Sucrose	684
Casein with added vitamins[a]	240
Salt mixture[b]	26
Corn oil with added vitamins[c]	50

[a] *Casein with added vitamins.*
To each 100 gm. of casein (De-Vitaminized Casein from Sheffield Chemical Co., Norwich, N. Y.) add:

	mg.
Thiamine hydrochloride	1.46
Riboflavin	1.46
Pyridoxine hydrochloride	1.46
Nicotinic acid	10.4
Calcium pantothenate	8.3
Choline chloride	417.0
Inositol	4.7
Para-aminobenzoic acid	126.0

[b] *Salt mixture*
Mix the following salts:

	gm.
K_2HPO_4	645
$NaH_2PO_4 \cdot H_2O$	126
$MgSO_4$	100
$NaCl$	282
Ferric citrate · H_2O	55
KI	1.6
$MnSO_4 \cdot H_2O$	9.0
$ZnCl_2$	1.0
$CuSO_4 \cdot 5H_2O$	0.6

[c] *Corn oil with added vitamins*
To each 100 gm. of corn oil add:

β-Carotene	22 mg.
Irradiated ergosterol	6000 I.U.
2-Methyl-1,4-naphthoquinone	12 mg.
α-Tocopherol	100 mg.

taining 1 ml. of 2N hydrochloric acid. At the end of the collection period, the funnels of the cages were washed down with a stream of distilled water, and the combined urine and washings were diluted to 100 ml. with distilled water. The total urinary inorganic phosphate for the period was determined by the method of Fiske and Subbarow (4). The crude parathyroid extracts were prepared by extracting ground frozen bovine parathyroid tissue[3] with 10 times the weight of 0.1N hydrochloric acid for 15 minutes at the temperature of a boiling water bath. The extracts were standardized against Parathyroid Extract U.S.P.[3] by the assay method of Munson *et al.* (1, 2) for calcium-mobilizing activity. Two extracts were used in this study. One extract, P20, contained approximately 28 U./ml., and the other, P22, contained 36±8 U./ml.[4] The extracts contained a negligible amount of inorganic phosphate.

RESULTS

Comparison of Intact with Parathyroidectomized Rats and the Effect of the Duration of Collection of Urine. Male rats were maintained on the stock diet for 7 days after receipt from the supplier. At the time of the experiment they were 46 days old and 120–150 gm. in weight. They were randomly divided into two groups, parathyroidectomized and intact, and each group was further divided into two subgroups, control and injected. The control rats received no treatment. The injected rats were given 3.0 ml. of parathyroid extract P20 subcutaneously per 100 gm. body weight. The parathyroidectomized rats were injected immediately following the operation. Urine was collected individually from each rat for 24 hours after the injection in three consecutive periods, 6, 6, and 12 hours, respectively.

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[3] Generously donated by Eli Lilly and Co., through the courtesy of Dr. K. K. Chen.

[4] In this paper, a value following the ± sign without other designation denotes the standard error. A value followed by the abbreviation (s.d.) denotes the standard deviation.

The urinary excretion of inorganic phosphorus, in mg. per hour, for each period is shown in Figure 1. The untreated parathyroidectomized rats excreted phosphate at a much lower rate than the intact rats in all three periods. Injection of parathyroid extract was followed by a marked increase in urinary phosphate in both the intact and the parathyroidectomized rats, but for unknown reasons the increase was greater in the parathyroidectomized rats. The effect of the extract on urinary phosphate was greatest in the first 6 hours. The increase over the controls was considerably diminished in the second 6-hour period and was further diminished in

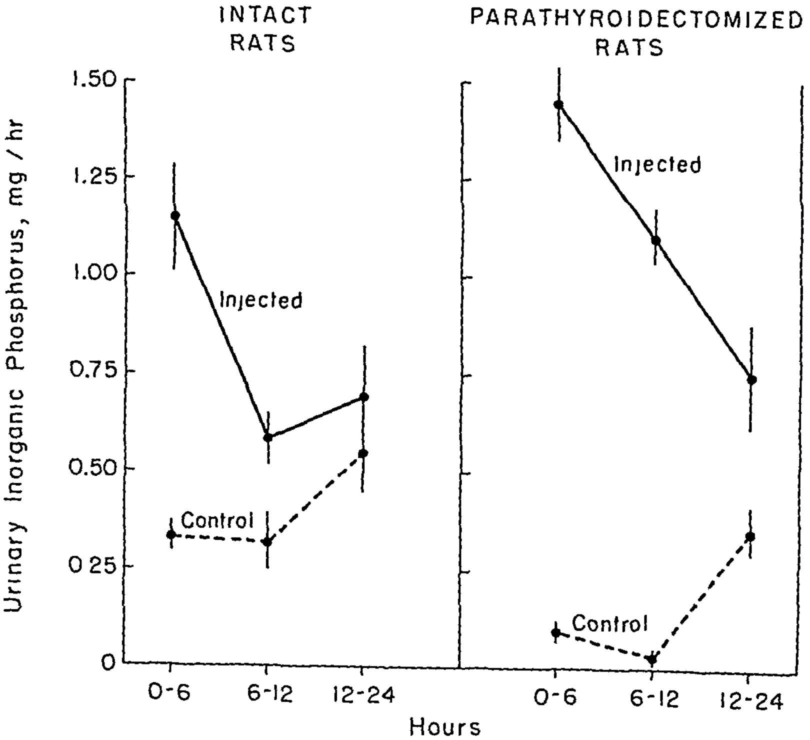

FIG. 1. Rate of urinary excretion of inorganic phosphorus by intact and parathyroidectomized rats. Parathyroid extract (84 units/100 gm.) was administered to the Injected rats; the Control rats were untreated. Each point (•) represents the mean of a group of 6 or 7 rats. The vertical line through each point represents the standard error.

the next 12 hours, although, at least in the parathyroidectomized rats, the difference between the injected and the control rats was still highly significant.

The increased rate of urinary excretion of phosphate by the untreated intact rats during the last 12 hours may indicate the beginning of a catabolic phase in the fasting animal. An additional factor contributing to the increase shown by the untreated parathyroidectomized rats during the same period may have been a marked rise in plasma inorganic phosphate resulting from the earlier retention of phosphate.

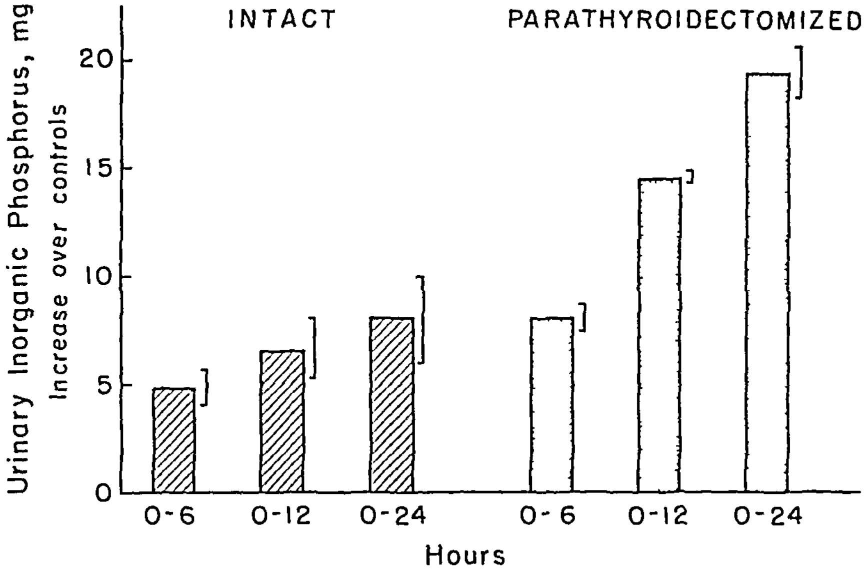

FIG. 2. Effect of parathyroid extract (84 units/100 gm.) on the cumulative urinary excretion of inorganic phosphorus by intact and parathyroidectomized rats. The height of each column represents the difference between the mean values for injected and control rats. There were 6 or 7 rats in each group. The brackets represent the respective standard errors.

The cumulative excretion of inorganic phosphorus by the injected rats for 6, 12, and 24 hours, after subtraction of the corresponding control value, is shown in Figure 2. Although the maximum increase in *rate* of phosphate excretion occurred during the first six hours, the *total effect* of the injection was greater when summated over the entire 24 hours, at least in the parathyroidectomized rats. The selection of a 6-hour period for collection of urine for the bioassay was therefore a matter of convenience rather than necessity, and it is acknowledged that a longer collection period might prove to be advantageous.

Standard Procedure for the Biological Assay Method. On the basis of the results of the preceding experiment a biological assay method was designed employing acutely parathyroidectomized male rats, eight to nine weeks of age. The rats were maintained on the stock diet for approximately one week after receipt from the supplier, and were then arranged in order of decreasing weight and assigned to treatment groups by formal randomization. They were next, in rapid succession, anesthetized with ether, parathyroidectomized, injected subcutaneously with parathyroid extract, and put into metabolism cages. The urine was collected for the following 6 hours. In the usual experimental design there were two dose levels of a suitable standard extract at a four-fold dose interval and as many unknowns as convenient, with 5 to 8 rats in each treatment group. The potency of an unknown was estimated in relation to the standard as described by Bliss (5) for parallel-line assays.

Results with the Standard Procedure. Eleven assays, using 232 rats, were conducted according to the standard precedure. The composite log dose-response curve of the 9 statistically valid assays is shown in Figure 3. In the remaining 2 assays the regression of the effect on the log dose of standard was not statistically significant. The mean index of precision (λ) of the 9 valid assays was 0.30 ±.083 (s.d.), and the minimum expected standard error of the estimated potency of an unknown when tested at 2 dose levels against 2 dose levels of standard, using 6 rats per group, was 33%.

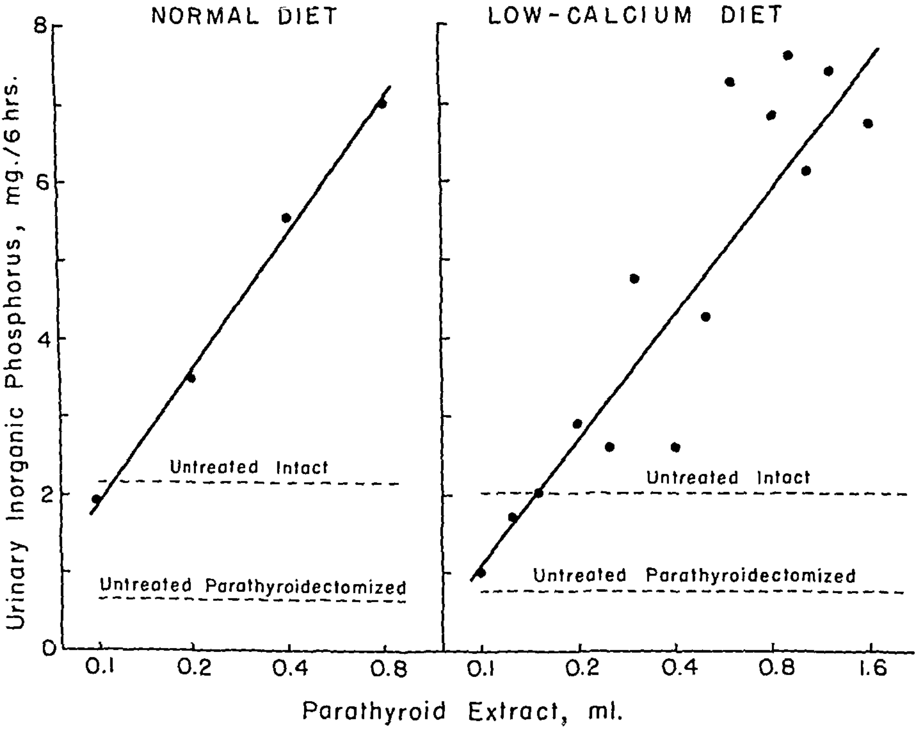

FIG. 3. Composite log dose-response curves for the biological assay methods for phosphaturic activity. Standard procedure (Normal Diet) on the left, modified procedure (Low-Calcium Diet) on the right. The horizontal dotted lines represent the average level of urinary excretion of inorganic phosphorus over a period of 6 hours for, respectively, intact and parathyroidectomized rats on the two diets. The standard errors of these control values ranged from 0.07 to 0.62 mg./6 hrs.

Results with a Modified Procedure. The biological assay method of Munson *et al.* (1, 2) for the calcium-mobilizing activity of parathyroid extract employs an experimental design identical with the standard assay procedure for phosphaturic activity except for the maintenance of the rats on a low-calcium diet for the four days immediately preceding the assay. With this modification, therefore, it is possible to conduct both assays on the same rats simultaneously. However, in a total of 36 simultaneous assays using 984 rats, the data on urinary phosphate provided only 22 valid assays. The composite log-dose response curve of the 22 valid assays is shown in Figure 3. The remaining 14 assays were invalid because of failure to achieve a statistically significant regression. The mean index of precision (λ) of the 22 valid assays by the modified procedure was 0.37 ± 0.15 (s.d.) as compared with 0.30 ± 0.08 (s.d.) for the standard procedure. Our preference for the standard procedure is based on both the possibly lower precision ($P < .2$) of the valid assays and the high proportion (39%) of invalid assays obtained with the modified procedure.

Completeness of Urine Collections. Although at the beginning of the urine collection period emptying of the bladder usually occurs during the anesthesia required for the operation, at the end of the 6-hour period the rats are removed from the metabolism cages without receiving any stimulus for micturition. Thus part of the observed variation between rats may have been due to incomplete collection of the urine. In one assay, by the modified procedure, the urine was analyzed for creatinine (6) as well as for phosphate. The assay statistics were computed in two ways, first, as usual, ignoring the values obtained for creatinine, and secondly, using the phosphorus to creatinine ratio. By the use of the phosphorus: creatinine ratio λ was apparently reduced, from 0.42 to 0.32. Although this change was not statistically significant, the ratio of the residual variances (F) was 2.14, with only 2.30 required for significance at the P.05 level. The results therefore suggest that incompleteness of urine collection may have contributed to the observed variation between rats and that the use of the phosphorus: creatinine ratio or deliberate manual emptying of the bladder might reduce the error of the assay.

Applications of the Assay Method for Phosphaturic Activity

1. Comparison of two parathyroid extracts for relative amounts of calcium-mobilizing and phosphaturic activity.

It was now possible, with the aid of the two biological assay methods, to compare the relative amounts of the two activities in various extracts. As reported previously (2, 7), our crude parathyroid extract, P22, was assayed twice for calcium-mobilizing activity and three times for phosphaturic activity. When the data were combined, P22 was found to contain $36 \pm 8\%$ of the calcium-mobilizing activity and $134 \pm 24\%$ of the phosphaturic activity of an equal volume of the commercial U.S.P. extract. The ratio of phosphaturic to calcium-mobilizing activities was 3.6 ±0.67 times as great in P22 as in the commercial extract.

2. Assay of spleen extract.

It has been suggested that because extracts of spleen and thymus were found to be phosphaturic in the dog that the phosphaturic activity of parathyroid extracts is not hormonal in nature (8). Ground, frozen bovine spleen was extracted with hot acid as described above for parathyroid extract. No phosphaturic activity could be detected in this spleen extract by our assay method in parathyroidectomized rats, and if any was present, it was less than $\frac{1}{16}$ that of crude parathyroid extract. There was also no detectable calcium-mobilizing activity in the spleen extract.

3. Assay of dialysates of parathyroid extract.

Various parathyroid extracts were subjected to equilibrium dialysis in cellulose tubing, $1\frac{3}{4}$ in. flat width, for 6 days at 4° C. and pH 2 (9). The original extract, the dialysand, and the dialysate were assayed for phosphaturic activity by the present method. A considerable proportion, approximately 20%, of the total phosphaturic activity of the extract was found in the dialysate. Calcium-mobilizing activity was also found in the dialysate, in contrast with the first report by Davies and Gordon (10) but in conformity with their later studies in which parathyroid extract was ultrafiltered through dialysis tubing (11). The ratio of the two activities in the dialysate was not significantly different from that in the dialysand or in the starting material.

DISCUSSION

The biological assay method for phosphaturic activity that we have described, although it falls somewhat short of the desired order of precision, appears to be more precise than alternative methods, recently reviewed by Greep and Kenny (12). Further possible improvements were noted in the Results section and are currently under investigation.

The modified assay procedure, described above, is conducted in conjunction with the assay for calcium-mobilizing activity, with the expenditure of little additional effort. Although the use of the modified procedure increases the chance of obtaining an invalid assay, it supplies data of at least a preliminary nature which, if sufficiently interesting, may be checked by a separate and more precise phosphaturic assay using rats maintained on a normal diet.

The specificity of the assay method for phosphaturic activity has not yet been studied extensively. For example, the importance of changes in renal hemodynamics during the collection of urine has not been assessed in relation to the effect on the tubular reabsorption of phosphate. Crawford *et al.* (6) state that ordinarily the contribution of increased glomerular filtration rate is of little importance in rats.

The suggestion by Stewart and Bowen (8) that the phosphaturic activity of parathyroid extracts is an artifact also found in extracts of thymus and spleen was not supported in the present investigation in which bovine spleen extract was found to contain no detectable phosphaturic activity in rats. The thoroughly documented observations that parathyroidectomy is followed by retention of phosphate and that administration of parathyroid extract results in an increased urinary excretion of phosphate certainly favor the existence of a phosphaturic parathyroid hormone. It is possible, however, that not all of the phosphaturic activity of parathyroid extracts is hormonal in nature.

The factor of 3.6 between the ratios of phosphaturic to calcium mobilizing activity in a crude parathyroid extract and in a commercial U.S.P. preparation (2, 7) has been confirmed by Davies *et al.* (13). The possible significance of this discrepancy in ratios for current experience (14, 15, 16, 17, 18) with the Ellsworth-Howard test (19) has been discussed elsewhere (2, 12). Differences in the ratios of the two activities in different extracts would indeed not be surprising if the parathyroid contained two hormones. Proof of this interesting hypothesis will require chemical separation and purification of two hormones. One (20) of three recent reports on purified parathyroid preparations states that both phosphaturic and calcium-mobilizing activities were present. There are other possible explanations of the discrepancy in ratios. First, the crude extract may contain nonhormonal phosphaturic material. Secondly, as noted by Rasmussen and Westall (21), the difference between the two extracts may be pharamacological in nature, since the assay for calcium-mobilizing activity is based on a measurement at a certain instant of time while the assay for phosphaturic activity is based on a cumulative response. Whatever the cause of the difference in assay ratios, a real difference in hormone content, the variable presence of artifact, or a difference in duration of action, caution should be exercised in comparing the phosphaturic activities determined by different assay methods. The definition of a unit of phosphaturic activity in terms of a standard assay method or, preferably, the establishment of a reference standard would tend to minimize the problem.

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THE EFFECTS OF COBALT INJECTIONS ON TOTAL CIRCULATING RED CELL VOLUME AND BONE MARROW CYTOLOGY IN NORMAL AND ADRENALECTOMIZED DOGS

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ABSTRACT

Adrenalectomized and intact female dogs were injected with cobalt alone for 30 days. Adrenalectomized dogs were injected for an additional period with a combination of cobalt and hydrocortisone. At 10-day intervals measurements were made of the hematocrit, hemoglobin, red cell counts and body weight. Total circulating red cell volume (P^{32} method) was measured at the end of and at various intervals during the course of the experiment. Femoral bone marrow myelograms and total nucleated cellularity were determined on the day following the last injection in each dog.

Intact dogs responded to cobalt injections with a significant increase in hematocrit, red cell count, hemoglobin, red cell volume and erythroid precursors within femoral bone marrows, while the blood picture and bone marrow cytology of adrenalectomized dogs injected with cobalt were not significantly different from those of desoxycorticosterone-maintained adrenalectomized dogs. Treatment of adrenalectomized dogs with a combination of cobalt and hydrocortisone tended to suppress the myeloid hyperplasia seen in the bone marrows of DCA-maintained adrenalectomized dogs. These data are interpreted to mean that an adequate supply of adrenocortical hormones is necessary for the hematopoietic response to cobalt.

WE HAVE reported previously that the adrenalectomized rat does not respond to cobalt injections with a characteristic polycythemia, as do sham operated controls, until they develop accessory adrenals (1). Stewart and Rogoff (2) reported that only one of 150 adrenalectomized dogs was found with an accessory adrenal at autopsy. Therefore, the dog appears to be a more reliable test animal than the rat for evaluating the effects of adrenalectomy on cobalt polycythemia over a prolonged interval provided adequate maintenance therapy is instituted immediately after operation.

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In view of these facts and to further clarify the role of the adrenal gland in cobalt polycythemia, a comparison has been made of the effects of intraperitoneal injections of cobalt on the total circulating red cell volume and nucleated erythrocytes of the bone marrow of normal and adrenalectomized dogs.

MATERIALS AND METHODS

Twenty healthy adult female mongrel dogs were used in these studies. They were divided into 4 groups as follows: Group (1) 5 intact untreated, (2) 7 intact dogs given 1.0 mg. elemental cobalt per kg./day i.p., (3) 4 adrenalectomized DCA maintained controls, (4) 4 adrenalectomized DCA maintained dogs receiving 1.0 mg. elemental cobalt per kg./day, two of which were continued for an additional period of time on a combination of 1.0 mg. elemental cobalt and 7 mg. hydrocortisone acetate per kg./day.

Daily rations for all intact dogs consisted of 2 cans of dog food and occasional supplementation with Purina chow. Each dog received an additional supplement of one pound of ground beef twice a week. All dogs were conditioned on an animal farm at least 3 weeks prior to use in an experiment. They were subjected to deworming, distemper vaccination and treatment for mange at least 2 weeks before use. Blood was drawn from the unanesthetized dog without stasis from either the jugular or radial vein and delivered to a heparinized tube. Erythrocyte counts were done using U.S. certified blood pipettes and the improved Neubauer counting chamber. Hematocrit determinations were made with Wintrobe hematocrit tubes which were spun for 30 minutes at 3000 r.p.m. at a radius of 17.5 cm. Hemoglobin determinations were made on the Klett-Summerson Photoelectric Colorimeter using the acid hematin method (3). Each dog was weighed at the time of the determination of the various blood values.

The red cell volume was determined with P^{32} tagged erythrocytes by the method of Hevesy *et al.* (4) as modified by Krieger *et al.* (5). Ten ml. of a tagged red cell suspension was injected via jugular or radial vein of the unanesthetized dog. After allowing 10 minutes for the sample to mix, 4–5 ml. of blood was removed from the opposite vein for wet counting on an end window Geiger-Mueller counter. Cobalt chloride ($CoCl_2 \cdot 6H_2O$)[2] was given daily as an intraperitoneal injection at a dosage of 1.0 mg. elemental cobalt (4 mg. $CoCl_2 \cdot 6H_2O$) per kg. of body weight.

Bilateral adrenalectomy was performed on the fasted dog under Nembutal[3] anesthesia using aseptic technique. As soon as the first adrenal was removed, 5 mg. desoxycorticosterone acetate, 50 mg. cortisone acetate, and 600,000 units of aqueous procaine penicillin suspension were injected intramuscularly. Immediately following the removal of the second gland both incisions were rinsed with Zephiran[4] and 125 ml. of warm, sterile saline-glucose solution was injected into the jugular vein. The solution contained 0.9% NaCl plus 5% glucose. For 3 days following the operation 5 mg. desoxycorticosterone and 25 mg. cortisone per day were given intramuscularly. The cortisone was then discontinued and the dosage of desoxycorticosterone reduced by 1 mg. per day, until the dog was receiving 1 mg. per day. The adrenalectomized dogs were maintained on 1 mg. desoxycorticosterone per day i.m. plus 2 grams NaCl added to the ration each day. If at any time during the experiment the dogs refused food on two consecutive days, 50 mg. cortisone and 5 mg. desoxycorticosterone were administered i.m. and the dosage of DCA gradually reduced over the next few days. Body ulcers developing in the long term adrenalectomized dogs were successfully combated with sulfadiazine ointment. At the end of the study the adrenalectomized dogs were autopsied and examined for evidence of accessory adrenals. P values were determined by using Fisher's "t" test for statistical significance.

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[2] Analytical grade $CoCl_2 \cdot 6H_2O$ with maximum limits of impurity for lead of 0.005%.

[3] 0.5 ml./kg. Abbott Veterinary Nembutal —60 mg./ml.

[4] Winthrop-Stearn Zephiran Chloride solution diluted 1:1,000.

Bone Marrow Technique.—The dogs were killed with ether and immediately after death the lower three fourths of the femur was dissected and sawed free on both sides. The femurs were placed in a vise and a one quarter inch cross section 25 mm. from the terminal end sawed off and placed in zenker-formol fixative to be prepared for differential cell count. The remaining part of the femur was used for the total nucleated cell count. The total nucleated cell count was done on the fresh marrow squeezed out of the terminal end of each femur. Two successive 120 mg. samples of bone marrow from each femur were weighed on a watch glass and quantitatively transferred to a ground glass homogenizer and diluted with 3 ml. of 2% acetic acid solution. After homogenizing the bone marrow suspension, a sample was transferred to a counting chamber with a red cell pipette and a count made from the 4 corner squares.

Calculations.—The average of the 4 bone marrow samples was inserted into the following formula:

$$\text{No. Nucleated Cells per mg.} = \frac{\text{Average No. Nucleated Cells in 4 squares}}{4} \times 10 \times \frac{3000}{120}.$$

The 3 ml. dilution factor was found to be an acceptable dilution for hypoplastic, hyperplastic and bone marrow of normal cellularity. The bone marrow section for differential cell counting after fixation in zenker-formol solution was washed, decalcified in formalin and trichloroacetic acid, dehydrated in alcohol, cleared in chloroform, embedded in paraffin, sectioned at 5μ, mounted and stained with giemsa. Five hundred cells were differentiated from the finished preparation with a lined square grid in the eye piece under oil immersion lens.

RESULTS

Peripheral Blood.—Changes in body weight, hematocrit, red cell count, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and total circulating red cell volume after cobalt injections in normal and adrenalectomized dogs are shown in table 1.

Cobalt injections in the nonadrenalectomized dog (Table 1) induced a significant elevation in hematocrit, red cell count and hemoglobin. After 30 days (15 days in one animal) of treatment the hematocrit had increased from 42.4 ±3.6 to 52.1 ±2.5%, ($P<.01$); the erythrocyte count, from 6.7 ±.87 to 9.2 ±1.5 million cells per cu. mm.($P<.01$); the hemoglobin, from 15.5 ±1.4 to 17.3 ±1.3 gm. per 100 ml. ($P=.03$). The MCV was decreased from 64.5 ±5.1 to 58.0 ±7.9 cubic microns; however, this decrease in cell size was not significant ($P=.1$). The MCH and MCHC were decreased in most instances under cobalt treatment. The red cell volumes expressed as ml. RBC per kg. original body weight (Fig. 1) for the group of intact dogs receiving cobalt for 30 days had a mean value of 46.1 ±6.6 ml. per kg., which was significantly ($P<.01$) higher than the mean of 36.0 ±5.6 for nonadrenalectomized untreated dogs.

Cobalt injections failed to cause an elevation in blood values (Table 1) in adrenalectomized dogs as compared with cobalt treated nonadrenalec-

TABLE 1. THE BLOOD PICTURE OF INTACT AND ADRENALECTOMIZED FEMALE DOGS TREATED WITH COBALT OR A COMBINATION OF COBALT AND HYDROCORTISONE

Treatment	No. dogs	Day exp.	Body wt. (kg.)	Hemat.	RBC	HB	MCV	MCH	MCHC	Total RBC volume	Red cell volume (ml./kg.)
Nonadrenalectomized											
I. Untreated	5	0	12.0± .77	43.6±4.4	6.5± .90	15.2±1.4	67.1± 3.0	23.6±2.8	35.0±2.5		
	5	10	11.6± .94	41.6±2.6	6.4± .75	14.6± .86	65.7± 8.5	23.1±4.0	35.5±1.9		
	5	20	12.1±1.2	40.2±5.4	6.5±1.4	14.3±1.6	61.8± 8.8	22.0±3.0	35.6±1.4		
	5	30	12.5±2.1	40.6±4.0	6.0±1.2	14.3±2.2	68.7± 7.5	24.1±3.1	35.2±2.3	451.6±112.2	36.0±5.6
II. Cobalt*	7¹	0	12.7±2.0	42.4±3.6	6.7± .87	15.5±1.4	64.5± 5.1	23.4±2.7	36.2±2.2	447.7±114.0‡	38.9±4.4
	7	10	12.7±1.9	48.7±2.2	8.6±1.1	16.8±1.0	58.1±10.4	20.0±3.7	34.5±1.8		
	6	20	12.2±1.5	51.7±3.2	9.8± .88	17.8±1.4	53.2± 6.4	18.4±2.3	34.5±1.2		
	6	30	12.5±2.2	52.1±2.5	9.2±1.5	17.3±1.3	58.8± 7.9	18.9±2.5	32.8±2.2	590.0±131.0	46.1±6.6
Adrenalectomized DCA 1 mg./kg./day											
III. Untreated	4	0	13.4±2.4	42.8±3.3	7.6±1.6	14.7±2.0	58.5±15.2	19.9±3.9	34.4±2.3		
	4	10	12.4±1.6	38.8±6.5	6.6± .88	13.7±1.9	59.1±12.0	20.9±3.1	35.4± .97		
	3	20	11.7± .91	42.3±4.1	6.6± .90	13.6±1.9	64.8±16.6	20.7±2.4	32.1±2.9		
	2	30	11.0± .50	42.0±3.2	6.5± .64	13.7±1.6	65.0± 2.0	20.6± .30	32.5± .70		
	2	40	10.7± .70	35.5±4.9	5.7± .78	12.5±1.1	63.0± 0.0	22.2±1.2	35.5±1.8		
	1	50	12.5	28.0	6.0	10.4	46.5	17.4	37.0	348.7± 34.6§	29.7±2.6
IV. Cobalt*	2	0	11.6± .60	40.5±3.5	5.9± .60	12.9±2.4	68.5± 1.5	21.7±1.9	31.6±2.2		
	2	10	12.1± .30	31.0±1.0	4.0±1.3	9.9± .20	85.8±24.2	27.6±8.2	31.8± .60		
	2¹	20	12.8±1.1	32.0±0.0	6.7± .90	11.3±1.2	48.5± 6.5	17.4±4.2	35.1±3.0	369.7± 24.1	29.3±4.3
	2	30	11.6± .60	34.0±3.0	5.2± .20	12.5±1.4	65.3± 3.2	23.9±1.9	36.7± .90		
	2	40	11.6± .10	30.0±4.0	4.3± .20	11.1±1.4	71.0±12.0	26.2±4.2	37.1± .30		
	2	50	11.7± .20	29.5±2.5	4.3± .40	9.8± .90	68.8± .80	22.8±0.0	33.2± .20	318.9± 21.2	27.4±2.4
V. Cobalt* Hydrocortisone†	2	0	14.1± .30	43.0±3.0	8.0± .40	15.3±1.1	54.0± 6.5	19.3±2.3	35.5± .10		
	2	10	13.8±2.0	33.0±3.0	5.6±1.3	11.6±2.0	61.0± 9.0	20.8±1.2	34.7±3.1		
	2	20	13.6±1.2	36.0±4.0	6.0± .90	14.3±1.1	61.0± 2.0	24.1±1.7	39.7±1.5		
	2	30	12.1±2.1	43.0±7.0	6.2±1.0	15.9±2.6	69.3± .20	25.6± .20	36.9± .10		
	2¹	40	11.2±2.1	39.0±7.0	6.0± .40	14.3±3.1	64.0± 8.0	23.5±3.7	36.4±1.4	328.1± 38.5	29.9±2.3
	2	50	11.1±1.1	38.5±7.5	6.0± .40	13.9±1.7	65.0± 7.0	22.8±1.6	36.5± .50		
	2	60	9.8±1.4	37.0±1.0	5.5± .10	13.2± .50	66.8± 3.7	23.6± .10	35.6±1.8		
	2²	70	8.9± .70	31.0±2.0	5.1± .70	10.0±0.0	62.0± 4.0	20.2±2.6	32.5±2.0	318.9± 32.5	34.4±2.2
	2	80	9.4±1.7	30.0±9.0	4.9±1.7	9.4±3.1	62.8± 2.7	19.5± .20	31.0±1.0		
	1	90	9.5	32.0	4.1	11.4	78.0	27.8	35.6		
	1	100	10.6	30.0	5.1	11.2	59.0	22.0	37.4		
	1	110	10.1	34.0	6.6	12.5	51.5	19.0	36.8		
	1	117	11.6	34.0	5.6	12.0	60.5	21.4	35.2	359.1	35.6

DAY = *Day of* experiment, BW = Body weight in kg., HEMAT = Hematocrit in per cent, RBC = Red *Cell* Count *in millions* per cu. mm., HB = Hemoglobin *in* g. per *100 ml.*, MCV = Mean corpuscular volume in cu microns, MCH = Mean corpuscular hemoglobin in micromicrograms, MCHC = Mean Corpuscular Hemoglobin Concentration in per cent, Total Red Cell Volume expressed in ml., ± = Standard Deviation.

* 1.0 mg. elemental cobalt/kg./day i.p.

† 7.0 mg. hydrocortisone/kg./day i.m.

‡ Mean initial red cell volumes for 3 of the 7 dogs.

§ = Mean red cell volumes for 3 dogs 20, 40 and 50 days respectively after adrenalectomy.

¹ = Cobalt chloride injections started i.p.

² = Cobalt chloride injections continued i.p. in addition to hydrocortisone i.m.

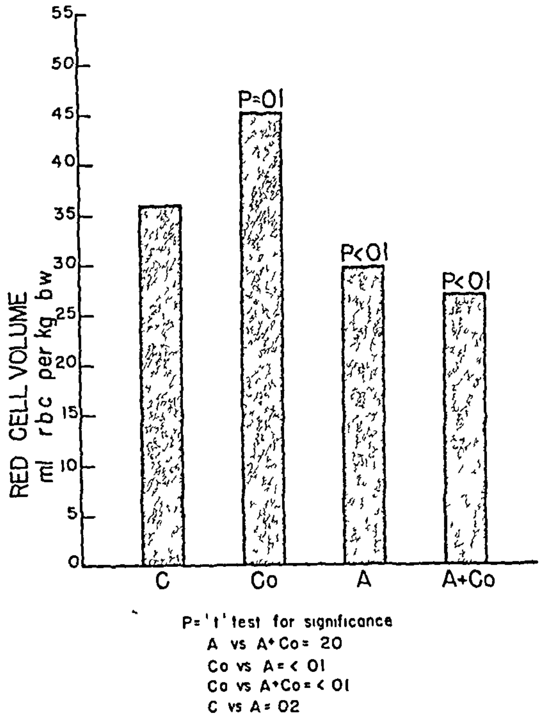


FIG. 1. Mean values for total circulating red cell volume expressed as ml. RBC per kg. body weight in intact untreated dogs (c), intact dogs treated with cobalt for 30 days (Co), DCA maintained adrenalectomized dogs (A) and DCA maintained adrenalectomized dogs treated for 30 days with cobalt (A+Co). P values over each bar indicate the significance in comparison with intact untreated dogs (c).

tomized dogs. In fact the blood values of cobalt-treated adrenalectomized dogs showed a progressive decrease as in adrenalectomized DCA maintained dogs. After 30 days of cobalt treatment in 4 adrenalectomized dogs (Table 1, Groups IV and V), the hematocrit had decreased from 35.5 ±7.0 to 30.0 ±2.8%; the erythrocyte count from 6.4 ±.88 to 4.7 ±.77 million cells per cu. mm.; the hemoglobin, from 12.8 ±3.2 to 10.0 ±.77 gm. per 100 ml. The mean MCV value for the 4 adrenalectomized dogs treated for 30 days with cobalt was 65.4 ±5.2 as compared with 67.1 ±3.0 for untreated nonadrenalectomized dogs and 64.8 ±16.6 for DCA maintained adrenalectomized dogs 20 days after adrenalectomy. MCH values in adrenalectomized dogs treated with cobalt for 30 days were decreased in most instances while the MCHC was not significantly different from untreated nonadrenalectomized or DCA maintained adrenalectomized dogs. The red cell volume in 4 adrenalectomized dogs receiving cobalt for 30 days (Fig. 1) was 27.0 ±3.3 ml. per kg. and significantly less ($P<.01$) than the mean of 36.0 ±5.6 for 5 intact, untreated dogs but not significantly different ($P=.2$) from the value of 29.6 ±3.2 for 7 DCA-maintained adrenalectomized dogs prior to treatment. A slight loss in body weight was noted in adrenalectomized dogs being treated with cobalt.

Two of the 4 adrenalectomized dogs (Table 1, Group V) treated for 30 days with cobalt were continued for an additional period on a combination of 1.0 mg. elemental cobalt as $CoCl_2 \cdot 6H_2O$ and 7 mg. hydrocortisone acetate per kg./day. One dog treated for an additional 57 days with the combination showed a moderate elevation in hematocrit (17%), erythrocyte

TABLE 2. DIFFERENTIAL BONE MARROW CELL COUNTS (MYELOGRAMS) IN INTACT AND ADRENALECTOMIZED DOGS TREATED WITH COBALT OR A COMBINATION OF COBALT AND HYDROCORTISONE (VALUES EXPRESSED AS PER CENT OF TOTAL CELLS ENUMERATED)

Treatment	No. dogs	Erythroid			Myeloid			Stem	Erythroid: myeloid ratio
		Early	Late	Total	Eosinophiles	Neutrophiles	Total		
Intact									
Untreated	8	7.8 ±1.9	33.7 ±3.4	41.5 ±3.0	2.4 ±1.4	55.0 ±3.4	57.3 ±3.2	1.2 ± .90	0.73 ± .09
Cobalt*	3	8.7 ±3.9	55.8 ±8.5	64.5 ±6.2	2.2 ±1.0	32.1 ±2.8	33.0 ±5.6	1.3 ± .90	1.94 ± .51
Adrenalectomized—DCA 1 mg./kg./day									
Untreated†	3	2.9 ±1.4	13.3 ± .65	16.2 ± 1.6	4.7 ±3.2	77.4 ± 5.6	82.1 ± 2.7	1.7 ±1.8	.20 ± .025
Cobalt*	2	1.2 ± .10	12.3 ± 2.9	13.5 ± 3.1	.8 ± .50	84.7 ± 1.8	85.6 ± 2.3	.8 ± .87	.16 ± .042
Cobalt‡ Hydrocortisone	2	8.4 ±3.2	25.8 ±10.3	34.1 ±13.4	0	62.8 ±14.7	62.8 ±14.7	3.2 ±1.8	.59 ± .035

* Cobalt Chloride at a dosage of 1.0 mg. elemental cobalt per kg./day for 30 days (i.p.)

† DCA maintained for 20, 40 and 50 days respectively. ± =Standard Deviation.

‡ Cobalt Chloride (1.0 mg. elemental cobalt/kg./day for 30 days i.p.) and a combination of cobalt chloride and hydrocortisone (7 mg./kg./day i.m.) for 57 days in one dog and for 20 days in a second dog.

count (27%), hemoglobin (20%) and total circulating red cell volume (2%). A second dog treated for an additional 20 days with the combination, with blood values being determined only after 10 days, also showed an elevation in hematocrit (18%), erythrocyte count (14%) and hemoglobin (25%).

An additional 5 intact dogs were injected with 2.5 mg. elemental cobalt per kg./day i.p. and none survived longer than 12 days. Two intact dogs were injected with 1.0 mg. elemental cobalt per kg./day subcutaneously and after 8–10 days necrosis and sloughing of the skin were noted at the injection site. The lack of toxicity of the lower dosages of cobalt when given via an intraperitoneal injection is apparently the result of its being spread out over a larger surface area, resulting in a more rapid absorption from the injection site. Three adrenalectomized dogs were injected i.p. with a dosage of 2.5 mg. elemental cobalt per kg./day and none survived longer than 5 days. The adrenalectomized animal is known to be more susceptible to toxic agents than the intact animal (6). No adrenalectomized dog was found with accessory adrenals at autopsy.

A method is also given for quantitating total nucleated cellularity of bone marrow, which varies from other techniques previously reported for quantitatively assessing total cellularity of bone marrow. The small variation in counts among intact untreated dogs (Table 3) indicates that within limits this technique is a reliable method for estimating quantitatively total nucleated cells of bone marrow. A further estimate of the total myeloid, erythroid and stem cells of bone marrow can be made by determining the percentage of the various types of bone marrow cells from the corresponding bone marrow section. Such a technique seems useful in that myelogram percentages alone may accentuate unimportant relationships and yield no information concerning the actual numbers of cells present. Volumetric techniques are available for quantitating bone marrow cellularity but have the disadvantage of diluting the bone marrow tissue excessively when a fatty bone marrow is encountered.

Bone Marrow Cytology.—In order to understand the significant changes which occur in the periperal blood of the dog during the administration of cobalt or a combination of cobalt and hydrocortisone, the status of the bone marrow seemed of utmost importance. The results of a study of the myelograms and total nucleated cellularity of bone marrows taken from some of the dogs seen in Table 1 are shown in Tables 2 and 3. The total ertythroid percentages and erythroid: myeloid ratios for nonadrenalectomized dogs receiving cobalt (Table 2) were significantly greater ($P < .01$) and total myeloid percentages were significantly less ($P < .01$) than in nonadrenalectomized untreated dogs. As seen in Table 3, the significant increase ($P < .01$) in total nucleated cells from the bone marrow of nonadrenalectomized cobalt-treated dogs was due entirely to an increase in total erythroid cells with no apparent change in total myeloid cells. The

TABLE 3. THE EFFECTS OF COBALT AND A COMBINATION OF COBALT AND HYDROCORTISONE ON THE TOTAL NUCLEATED CELLS OF THE BONE MARROW OF NORMAL AND ADRENALECTOMIZED DOGS

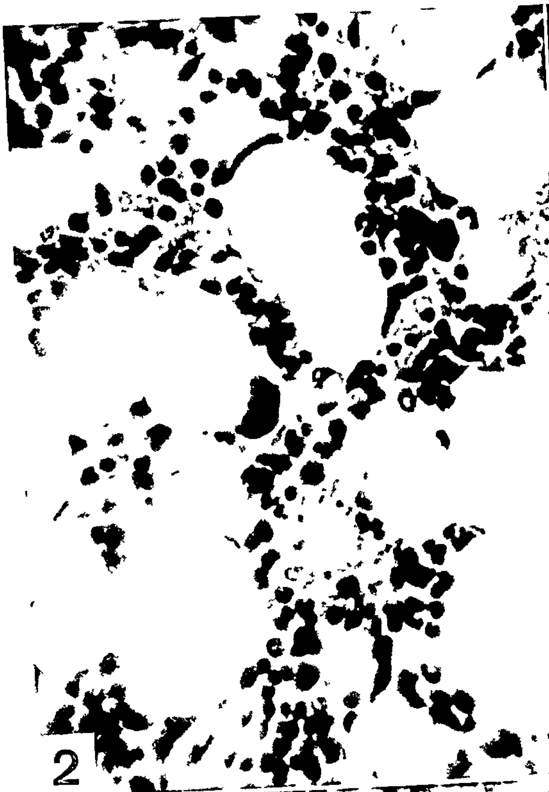
Treatment	No. dogs	Total nucleated cells	Total erythroid cells	Total myeloid cells	Total stem cells
		Thousands per mg. bone marrow tissue			
Intact					
Untreated	6	34.2 ± 3.8	14.1 ±1.9	19.6 ±2.3	.5 ± .6
Cobalt*	5	59.4 ±18.2	38.6 ±8.1	19.4 ±4.7	1.2 ± .88
Adrenalectomized—DCA/mg./kg./day					
Untreated	3	43.8 ± 9.8	7.1 ±1.9	36.1 ± 8.6	.51 ± .49
Cobalt*	2	39.6 ±22.8	5.0 ±1.9	34.2 ±20.4	.90 ± .32
Cobalt† + Hydrocortisone	2	24.6 ± 8.4	7.5 ± .97	16.0 ± 8.7	1.19 ± .60

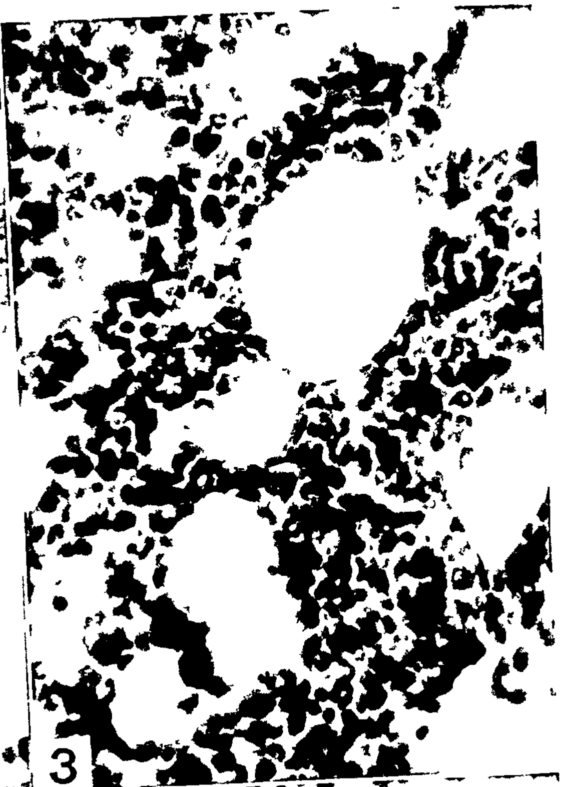
* Cobalt Chloride at a dosage of 1.0 mg. elemental cobalt per kg./day for 30 days (i.p.).
† Cobalt Chloride (1.0 mg. elemental cobalt/kg./day for 30 days i.p) and a combination of cobalt chloride and hydrocortisone (7 mg./kg./day i.m.) for 57 days in one dog and for 20 days in a second dog.
± = Standard Deviation.

increase in total erythroid cells was almost exclusively due to the late erythroid series or the orthochromatic normoblast as seen in Figure 3. The total stem cells were also increased in nonadrenalectomized dogs treated with cobalt.

DCA-maintained adrenalectomized dogs had bone marrows with a significant ($P < .01$) increase in total myeloid cells (Table 3) which was due largely to an increase in neutrophilic metamyelocytes, bands and segmenters. The observations on the bone marrow sections reflect changes observed in the myelograms (Table 2). Thus, a decrease in the erythroid elements and an increase in the relative numbers of myeloid cells shown in tables 2 and 3 are even apparent from a gross inspection of Figure 4. A significant decrease in the erythroid: myeloid ratio ($P < .01$) occurred in bone marrows from DCA-maintained adrenalectomized dogs. No significant change occurred in the relative numbers of stem cells in bone marrows from long term DCA-maintained adrenalectomized dogs.

Two DCA-maintained adrenalectomized dogs treated for 30 days with cobalt had bone marrow myelograms and total number of nucleated cells which were not significantly different from those of DCA-maintained adrenalectomized dogs. These findings confirm the total circulating red cell volume data showing that cobalt does not stimulate erythropoiesis in the adrenalectomized dog. The total number of erythroid cells and erythroid: myeloid ratios (Tables 2 and 3) appeared to be lower in bone marrows from adrenalectomized dogs treated with cobalt (Fig. 5) than those of DCA-maintained adrenalectomized dogs but the differences were not significant.





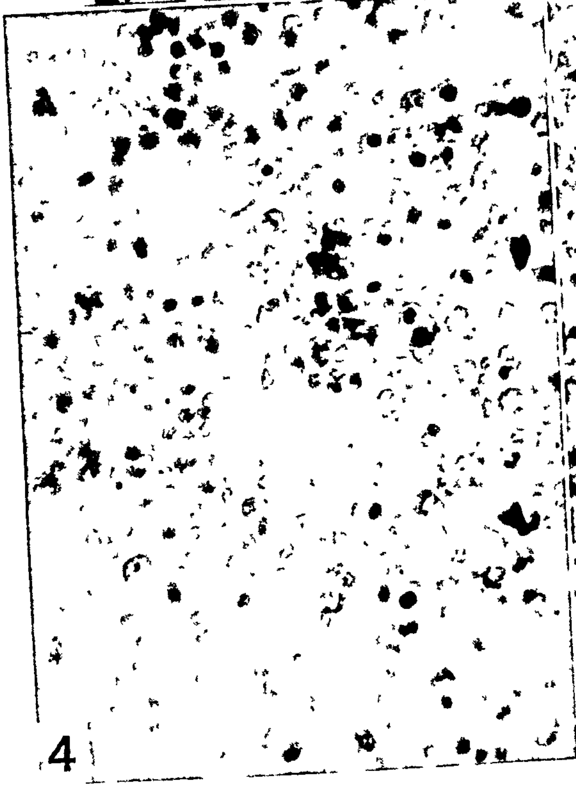


FIG. 2. Femur marrow from a nonadrenalectomized untreated dog ×143. Note moderate amount of fat cells and a few dark islands of cells representing erythroid elements.

FIG. 3. Femur marrow from a nonadrenalectomized dog treated daily for 30 days with

Two adrenalectomized dogs that had been treated for 30 days with cobalt and had shown no significant change in red cell volume were continued for an additional period (57 days in one and 20 days in a second dog) on a combination of cobalt and hydrocortisone. As seen in Table 1 an elevation in hematocrit, hemoglobin and red cell count resulted along with a slight to moderate increase in total circulating red cell volume. Both dogs showed some change (Tables 2 and 3) in the total number of myeloid cells after treatment with the combination. The results in Table 2 show an increase approaching a return to normal of the erythroid: myeloid ratio in the bone marrows taken from adrenalectomized dogs treated with both cobalt and hydrocortisone concurrently. Another point which desreves attention is the reappearance of the vacuolated fat cells (Fig. 6) in the bone marrow of an adrenalectomized cobalt-treated dog injected for an additional period with a combination of cobalt and hydrocortisone.

DISCUSSION

Some investigators of cobalt polycythemia have concluded that the normal dog, when given cobalt orally, fails to respond with a significant elevation in blood values (7, 8) similar to other species (9, 10, 11, 12). Brewer (13) statistically analyzed the results of several cobalt feeding experiments and found that a significant increase in red cell count, hemoglobin and hematocrit did occur when the daily oral dose exceeded 15 mg. per kg. Evidence is presented which establishes proof that the intact dog will respond to intraperitoneal injections of cobalt with a marked elevation in total circulating red cell volume. The increase in total erythroid cells and erythroid:myeloid ratios of bone marrow from intact dogs treated with cobalt confirm the elevation in total circulating red cell volume and are further proof that cobalt is capable of stimulating hematopoiesis in normal dogs.

It has been found that the adrenalectomized rat shows a moderate anemia for the first 20 days after adrenalectomy (14, 15, 16) which is corrected upon the development of accessory adrenals (16). The rat recovers from the anemia of adrenalectomy after 30 days (16), whereas the DCA maintained adrenalectomized dog still remains anemic 50 days after adren-

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1.0 mg. elemental cobalt per kg. ×143. Note increased cellularity along with the greater concentration of dark staining erythroid islands. Also note smaller number of vacuolated fat cells in comparison with the normal control in Figure 2.

FIG. 4. Femur marrow from adrenalectomized—DCA (1.0 mg./kg./day) maintained dog 22 days after adrenalectomy ×143. Note the markedly increased myeloid cellularity and complete disappearance of erythroid islands.

FIG. 5. Femur marrow from an adrenalectomized dog maintained for 20 days on 1.0 mg. DCA/kg./day and treated for the next 30 days with 1.0 mg. DCA and 1.0 mg. elemental cobalt/kg./day ×200. Note increased myeloid cellularity and lack of definitive erythroid islands.

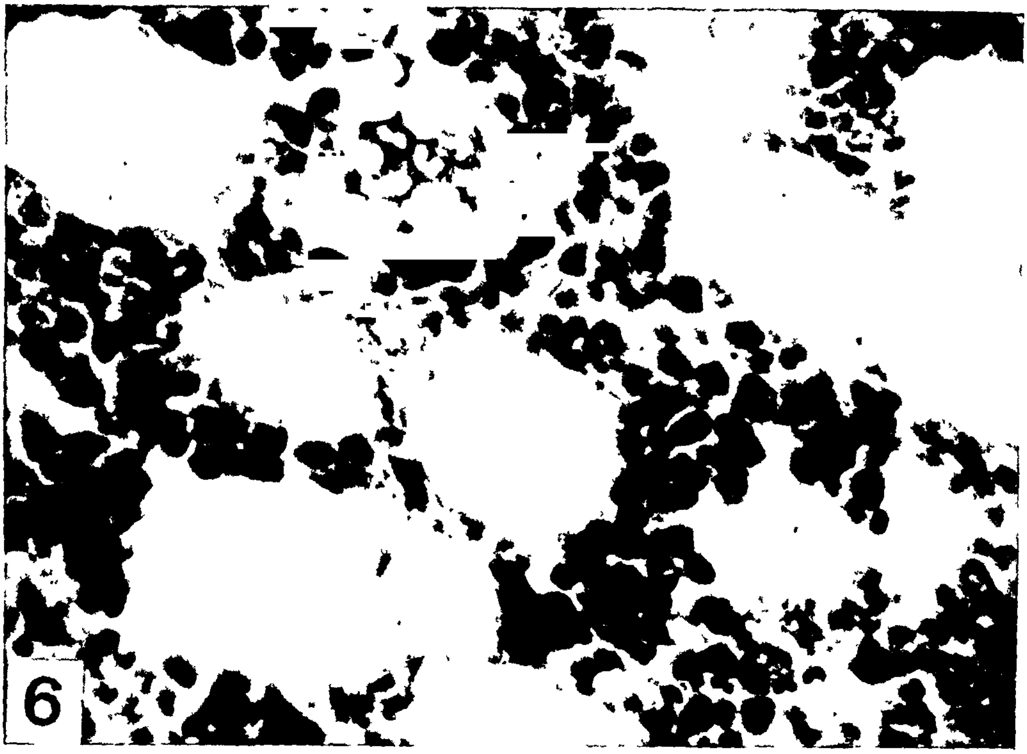

FIG. 6. Femur marrow from an adrenalectomized dog maintained for 40 days on 1.0/mg. DCA/day, treated for the next 30 days with 1.0 mg. DCA and 1.0 mg. elemental cobalt per kg./day and the next successive 57 days with DCA, cobalt and 7 mg. hydrocortisone per kg./day × 143. Note return of the normal appearance of erythroid islands and vacuolated cells similar to the normal dog in Figure 2.

alectomy and will go into severe adrenal insufficiency and die within a few days if the DCA injections and dietary salt supplementation are withdrawn. The failure of the adrenalectomized dog to develop accessory adrenals may explain the inability of the dog to correct the anemia of adrenalectomy. The red cell volume in adrenalectomized dogs with or without cobalt is significantly less than those of intact untreated dogs. The difference in red cell volumes in DCA maintained adrenalectomized dogs and adrenalectomized dogs treated for 30 days with cobalt is not significant, thus indicating that the removal of the adrenals in dogs renders them incapable of responding to cobalt injections with an elevation in blood values.

Swingle *et al.* (24) have reported that the low values for hemoglobin, hematocrit and erythrocyte count which characterize adrenalectomized dogs, in some instances, are increased to normal by long continued massive doses of cortisone. Administration of hydrocortisone or corticosterone to normal rats over a prolonged period of time induced a significant elevation in total circulating red cell volume (17) and the elevation in red cell volume achieved in adrenalectomized rats treated with concurrent injections of cobalt and hydrocortisone was significantly greater than the additive effects of either substance injected alone (18). Hydrocortisone and cobalt, when injected concurrently into dogs, improved the hematocrit, hemoglobin, erythrocyte count and total red cell volume to some degree. It seems quite likely that a favorable combination of cobalt and hydrocortisone can be found in which the "synergistic" action of the two drugs can be demonstrated to the same degree as with adrenalectomized rats.

The failure of adrenalectomized dogs to respond to cobalt injections with a change in red cell volume is interpreted to mean that the adrenocortical hormones are necessary for the production of the characteristic cobalt polycythemia. The fact that hydrocortisone and corticosterone are capable of stimulating erythropoiesis in the intact rat (17) and that a joint hematopoietic action of a combination of cobalt and hydrocortisone is exerted (18) seems proof that the action of cobalt and the adrenocortical secretions in stimulating erythropoiesis in synergistic.

The decrease in the erythroid: myeloid ratio and increase in total myeloid cells in bone marrow from DCA-maintained adrenalectomized dogs are similar to the findings of Gordon *et al.* (19) for the adrenalectomized rat. Fruhman *et al.* (20) found that the administration of corticosterone to adrenalectomized rats resulted in a significant increase in the number of nucleated erythroid cells and a decrease in the number of neutrophiles and eosinophiles within femoral bone marrows. The total erythroid and myeloid percentages for the intact untreated dog shown in Table 2 are very similar to bone marrow myelograms from 187 normal adult dogs reported by Albritton (21). Cobalt injections in adrenalectomized dogs induced no significant change in bone marrow myelograms. However, injections of a combination of cobalt and hydrocortisone into adrenalectomized dogs partially restored to normal the erythroid:myeloid ratio and the total nucleated cellularity of the bone marrow.

The evidence seems to suggest that cobalt does not produce polycythemia by a direct effect on bone marrow itself (22) but acts secondarily through an elevation in plasma erythropoietin (23). The most reasonable hypothesis at this time, to explain the role of the adrenal secretions in cobalt polycythemia, is that the adrenocortical hormones act synergistically with erythropoietin to stimulate the blood forming organs.

Acknowledgments

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CORTICOSTEROIDS AND THE FUNCTIONAL CAPACITY OF THE RAT HEART-LUNG PREPARATION[1,2]

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ABSTRACT

A technic has been developed for the preparation and maintenance of the isolated rat heart-lung. Index of left ventricular work expressed as cardiac output times mean arterial blood pressure was highly reproducible for a given set of experimental conditions. Order of work performance arranged from highest to lowest was as follows: 1) heart-lung from intact rat perfused with blood from intact rats, 2) heart-lung from adrenalectomized rat perfused with blood from intact rats, 3) intact-adrenalectomized, 4) adrenalectomized-adrenalectomized. Corticosterone, but not Substance S, added to the perfusion blood of the intact-adrenalectomized preparation restored work capacity toward that of the intact-intact preparation.

FUNCTIONAL impairment of the cardiovascular system is a prominent feature of adrenal insufficiency (1, 2, 3, 4), the nature of which has been the subject of numerous investigations. *In vivo* studies suggest that the impairment involves reduction in the contractile force of the myocardium (5) as well as inadequacy of peripheral vascular functions (6). *In vitro* studies designed to more clearly define the influence of the corticosteroids on the heart have included the isolated perfused heart and the muscle strip. The diversity of results and the relatively high concentrations of steroids employed make it impossible to reach definitive conclusions as to the action of the secretions of the adrenal cortex on the myocardium (7). For example, Nasmyth (7) found a depressant action of the corticosteroids on guinea pig and rat hearts perfused by the Langendorf technic, whereas Tanz *et al.* (8) described a positive inotropic action of 9α-fluorohydrocortisone on the isolated cat papillary muscle and on the isolated perfused rat heart.

The present study had two objectives: 1) to establish conditions for high reproducibility of work performance of the isolated rat heart-lung and 2)

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to examine the possibility that the corticosteroids have an influence on the functional state of this isolated preparation. Work performance has been selected as the index of functional state because of the relative ease with which it may be measured (pressure times cardiac output).

ANIMALS

Male rats, 300 to 350 gm. in body weight, were obtained from the Holtzman Farm. Bilateral adrenalectomy was performed two to three weeks before the isolation of the heart-lung; control animals were subjected to sham adrenalectomy. The anesthetic for all surgical procedures was sodium pentobarbital, 40 mg./kg., administered intraperitoneally as a one per cent aqueous solution. Adrenalectomized animals were maintained post-operatively on 0.9% sodium chloride solution; controls drank tap water. Both groups were housed in a constant temperature room for at least two weeks before isolation of the heart-lung. They were fed Friskies dog chow *ad libitum*. Completeness of adrenalectomy was checked by inspection of the suprarenal area.

HEART-LUNG PREPARATION

The isolated rat heart-lung preparation was similar to that described by Knowlton and Starling for the dog (9). In the preparation to be described the lungs were collapsed and not functional but the pulmonary vessels were part of the circuit. Blood was conducted by gravity in a thin film over a stainless steel screen in an atmosphere of 95% oxygen, 5% carbon dioxide and collected at the bottom of the oxygenator[5] which served as the "venous reservoir." In a series of preliminary observations it was demonstrated that positive pressure respiration induced pulmonary edema which was a major factor in limiting the survival of the preparation. Extrapulmonary oxygenation significantly prolonged survival time and maintained concentrations of arterial and venous oxygen of approximately 15 and 12 volumes per cent, respectively.

The course of the blood in the system was in brief: right heart, pulmonary vascular bed, left heart, aorta, innominate artery, bubble flowmeter, screen oxygenator, coiled tube immersed in constant temperature bath (37° C), inferior vena cava and back to right heart.

A capillary mercury manometer was connected by a limb of a T-tube to the circuit just beyond the carotid artery catheter. A tunnel clamp was employed to adjust peripheral resistance and was located in the circuit immediately before the bubble flowmeter.[6]

The operational sequence for the conduct of an experiment was as follows. 1) The venous reservoir was charged with 50 ml. of fresh heparinized blood obtained from anesthetized donor animals. 2) The trachea of an anesthetized experimental animal was connected to a positive pressure respirator. 3) A midline incision was carried from the symphysis pubis through the manubrium. 4) The thoracic walls were retracted. 5) The right and left superior vena cavae, the left common carotid and both subclavian arteries securely ligated. 6) Two loose ligatures were placed under the right common carotid artery high in the neck. 7) One loose ligature was placed about the thoracic vena cava and another about the arch of the aorta just distal to the origin of the innominate artery. 8) Heparin sodium (1.5 mg.) was injected into the left renal vein. 9) The distal ligature under the right common carotid artery was tied. 10) A small artery clamp was placed on the innominate artery. 11) A catheter was inserted into the right carotid artery, advanced into the innominate artery and secured with a ligature. 12) Initial infusion pressure (venous pressure) was fixed at 10 cm. of water by adjustment of the level of blood in the reservoir. In rapid succession 13) the inferior vena cava was cannulated at the point where the cava passes through the diaphragm, 14) the aortic arch ligated, 15) the inferior vena cava catheter secured with a ligature, 16) stopcocks, one on the inflow, the other on the ouflow tubing, were opened, 17) artificial respiration was terminated.

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[5] Dr. Richard Eckstein kindly loaned us this modified Gibbon oxygenator which consits of a 2×12×6″ rectangular lucite container in which is mounted a stainless steel wire screen.

[6] The authors will supply details about the plastic tube connections upon request.

The procedures must be performed in a relatively uniform manner, in less than 10 minutes, and with minimal hemorrhage. After the stopcocks were opened, an initial period of instability, less than one minute in duration, characterized by cardiac arrhythmia and unstable arterial pressure, was succeeded by a stable period of variable length. During the stable period, mean arterial pressure was maintained at 80 mm. Hg in Exp. 1 and 110 mm. Hg in Exp. 2 by adjustment of the peripheral resistance clamp. During the stable period, cardiac output, mean arterial blood pressure, and temperature of venous blood were periodically recorded or adjusted.

Left ventricular work index was calculated as follows:

$$W = K\frac{(\text{C.O.})\ (P)\ (T)}{M},$$

where W = the left ventricular work index in gram-meters per 100 mg. dry heart weight, K = a constant composed of conversion factors, C.O. = cardiac output in ml. per min., P = pressure in mm. Hg, T = time in minutes that the preparation was able to maintain a predetermined level of blood pressure, and M = dry heart weight expressed in mg.[7]

RESULTS

In Table 1 are presented the results of Experiment 1 in which peripheral resistance was adjusted to maintain a mean arterial pressure of 80 mm. Hg. Three preparations were studied: 1) heart-lung from an intact rat perfused with blood from intact rats (intact-intact), 2) heart-lung from an adrenalectomized rat perfused with blood from adrenalectomized rats (adrenex-adrenex), and 3) heart-lung from an intact rat perfused with blood from adrenalectomized rats (intact-adrenex). The work index (work of the left ventricle during the period in which the preparation was capable of maintaining a pressure of 80 mm. Hg) was influenced by the secretions of the adrenal gland. The work index of the heart-lung from either adrenalectomized rats or intact rats was at the same low level when the perfusion blood was obtained from adrenalectomized rats. The results suggest that impairment of functional activity was a consequence of low titers of corticosteroids and/or epinephrine or of toxic factors in blood from adrenalectomized rats.

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[7] The calculated left ventricular work index is not the total work performed by the left ventricle. Not included is 1) the work performed in accelerating the blood in its passage from the ventricle into the aorta and 2) that part of the cardiac output which is directed into the coronary arteries. In the intact heart, coronary flow is usually a relatively small fraction of the total cardiac output. However, in the isolated heart-lung, coronary flow may represent a relatively high fraction of total cardiac output. We have no experimental data to evaluate changes in coronary flow in this study. For that reason we have described our calculation of cardiac performance as an "index."

TABLE 1. LEFT VENTRICULAR WORK INDEX OF RAT HEART-LUNG (EXP. 1)[1]

Type of preparation	Intact-Intact[3]	Adrenex-Adrenex[4]	Intact-Adrenex[5]
Number of experiments	12	12	6
Work Index[2] ±S.E.	444 ±56	126 ±31	136 ±45

[1] Mean arterial blood pressure = 80 mm. Hg. No glucose added.

[2] Expressed as gram-meters per 100 mg. dry heart weight.

[3] Intact-Intact = heart-lung preparation from intact rat perfused with blood from intact rats.

[4] Adrenex-Adrenex = heart-lung preparation from adrenalectomized rat perfused with blood from adrenalectomized rats.

[5] Intact-Adrenex = heart-lung preparation from intact rat perfused with blood from adrenalectomized rats.

The total work index does not reveal certain characteristics of the three preparations. For that reason, cardiac output against time has been plotted in Figure 1 for three typical experiments. The marked decrease in cardiac output of the heart-lung from the adrenalectomized rat is apparent at the beginning of the experiment. In the case of the intact-adrenex preparation the cardiac output is initially relatively high.

The results of Experiment 2 in which mean arterial pressure was maintained at 110 mm. Hg are presented in Table 2. An additional preparation, adrenex-intact was included. Glucose was added at the beginning of the experiment to the perfusion blood in a concentration of 100 mg. per 100 ml. At the higher level of pressure the work index of the intact-intact preparation was less than that of the same preparation operating at a pressure of

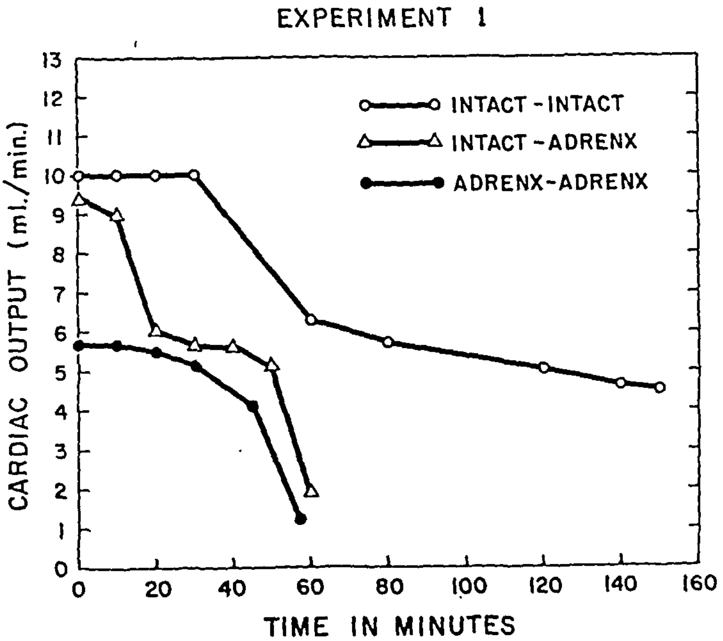

FIG. 1. Cardiac output plotted against time for three typical preparations in experiment 1. Area under each curve is related by a constant to left ventricular work index.

TABLE 2. LEFT VENTRICULAR WORK INDEX OF RAT HEART-LUNG (EXP. 2)[1]

Type of preparation	Intact-Intact[3]	Adrenex-Adrenex[3]	Adrenex-Intact[3]	Intact-Adrenex[3]		
Amount of steroid added per 100 ml. perfused blood	0	0	0	0	10 µg. Substance S	10 µg. Corticosterone
Number of experiments	18	15	15	15	5	5
Work Index[2] ±S.E.	235 ±24	25 ± 4	75 ±12	50 ± 5	49 ± 5	108 ± 6

[1] Mean arterial blood pressure = 110 mm. Hg, 100 mg. glucose added to each 100 ml. of perfusion blood.
[2] See Table 1 for legend.
[3] See Table 1 for legend.

80 mm. Hg. The impairment in function of the adrenex-adrenex preparation was even more marked than in Exp. 1. Perfusion of the heart-lung from adrenalectomized rats with blood from intact rats had a positive effect on work capacity. In contrast to Exp. 1, the intact-adrenex preparation performed a significantly greater amount of work than the adrenex-adrenex preparation.

Substance S (17α,21-dihydroxy-Δ^4-pregnene-3,20-dione) added to the perfusion blood of the intact-adrenex preparation induced no change in work capacity. On the other hand, corticosterone added in a concentration of 10 µg. per 100 ml., significantly improved the work index but did not restore it to normal.

DISCUSSION

Technics have been described for the preparation of the heart-lung in the rat (10, 11, 12, 13). In the study reported here, uniformity of surgical procedures, health of animals, extrapulmonary oxygenation, constancy of blood pressure, and maintenance of a constant temperature of the perfusion blood have led to the development of a heart-lung preparation which is characterized by a high degree of reproducibility of work performance.

The average cardiac output of the intact-intact preparations in Exp. 1 was 9.9 ml. per min., which is to be compared with 64 ml. per min. for cardiac output in normal rats measured by the direct Fick principle (14). This difference between the isolated heart and the heart *in vivo* in the rat is of the same order of magnitude as that observed in the dog. In the intact animal, coronary flow represents a small fraction of the total work of the left ventricle. In the isolated heart, coronary blood flow, which is not included in work index, may represent a significant fraction of the total cardiac output. Future studies should be directed toward measurement of coronary flow in the isolated rat heart in order to assess work capacity more precisely.

The basis of the impairment of function in the heart-lung deprived of the secretions of the adrenal is unknown. The following experiment suggests that one factor involved is a change in the functional state of the myocardium. Heart-lung preparations were prepared from intact rats and from rats adrenalectomized at various intervals prior to testing. Both types of preparations were perfused with blood from intact rats. The results indicate that a period of at least two weeks must elapse between adrenalectomy and testing before a significant reduction in work capacity can be demonstrated. Of interest is the fact that after two weeks of adrenal insufficiency myocardial atrophy as measured by dry weight (10% less than normal) is also definitely established. The mechanism of this myocardial atrophy which develops slowly after removal or destruction of the adrenals in the rat and in man (3) is unknown.

No data are available to evaluate the length of time after adrenalectomy which is necessary to manifest the changes in the perfusion blood which induce impairment of function of the heart-lung. The fact that addition of corticosterone in a concentration of 10 μg. per 100 ml. partially restores the functional capacity of the intact-adrenex preparation suggests that at least one acute factor is a deficiency of corticosteroids. However, a toxic factor or a deficiency of epinephrine may also contribute to the poor work performance of the intact-adrenex preparation.

Substance S was ineffective, corticosterone active, in the isolated system. Substance S has no effect on carbohydrate metabolism and has at most a slight influence on electrolyte excretion, whereas corticosterone significantly influences both types of metabolism in the whole animals. Additional studies are planned to relate activity of steroids in the whole animal and in the heart-lung.

The fact that certain corticosteroids are quite effective when administered by the oral route has prompted certain investigators (15, 16) to suggest that the liver exerts an "activating" influence on the corticosteroids. The present study indicates that the liver is not essential for the biological action of corticosterone.

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EXPERIMENTAL DIABETES IN THE CATFISH: NORMAL AND ALLOXAN-DIABETIC BLOOD GLUCOSE AND PANCREATIC HISTOLOGY[1]

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ABSTRACT

Alloxan diabetes has been demonstrated in the common lake catfish by correlated histological and blood sugar studies. Anatomical considerations make this animal particularly suitable for this work. The conus arteriosus is conveniently situated for intravascular injection and serial blood sampling. The concentration of endocrine pancreas in a principal islet and accessory islets, all with limited exocrine tissue, facilitates histological preparations. In 237 determinations, using a Folin-Malmros micro method modified for fish blood, the normal blood sugar value was found to be 59 mg.%, with a standard deviation of 19 mg.%. Alloxan was administered through the conus of 50 fish, at 400 mg./kg. In alloxanized fish, blood sugar levels of 120–200 mg.% were typical. This hyperglycemia persisted through the 96 hour period studied and, in a few fish not sacrificed earlier for histological study, for 14 days, the longest period studied. In the intact animal, the principal islet is composed of many, heavily-granulated beta cells, with a similar number of other islet cells and a cortex of exocrine tissue. Within 3 hours of alloxan injection, degranulation of beta cells was apparent. In subsequent sampling periods, up to 96 hours, degranulation increased, accompanied by progressive hydropic degeneration and nuclear disintegration. These changes were found only in beta cells.

THE mechanism of the diabetogenic action of alloxan has been investigated extensively but without conclusive results. The need for better understanding of this phenomenon has been increased by the Loubatiere hypothesis of the importance of endogenoüs alloxan in the etiology of human diabetes mellitus. It appears that special features of the lower vertebrates may facilitate observations pertinent to this hypothesis. These forms offer very large cells, favorable to histological or cytochemical study, and comparatively slow physiological responses, which may permit identification of functional changes too rapid for observation in mammals. Certain teleost fish provide the additional advantage of the concentration

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of islet tissue in nodules containing little exocrine tissue. They also provide convenient sites for injection and blood sampling, such as the gill arch of the toadfish or the conus arteriosus of the catfish.

Examination of the scanty literature of experimental diabetes in fish offers little encouragement. Reports are often based on very small numbers of animals and contain many conflicting observations. They agree on 2 points, the extreme variability of the normal blood glucose levels of fish and the comparable variability of response to alloxan.

Lazarow and Berman (8) have reported hyperglycemia and beta cell lesions in the toadfish, *Opsanus*, following alloxan injection. The observations of Nace (11), on the same form, have been less consistent. Grosso (3), using the guppy, *Lebistes*, has produced beta cell lesions in animals maintained in alloxan solutions. This careful and detailed histological study includes no blood sugar data and its interpretation is complicated by the observations of Patterson, Lazarow and Levy (15), of the very short effective life of alloxan under physiological conditions. While Lazarow and Berman mention only beta cell lesions, Grosso reports significant changes in liver, kidney and spleen.

In a series of 4 reports, Saviano (16) has described the effects of alloxan on 3 fish, *Scyllium*, *Motella* and *Scorpaena*. In none of these did he observe beta cell lesions comparable to those reported by Grosso and by Lazarow and Berman. In all 3 forms, alpha cell lesions were the most consistent feature. Hyperglycemia was found in some animals, but with no uniformity. In *Scyllium*, lesions of exocrine cells were a common observation. LaGrutta (7) has confirmed the findings of Saviano on *Scorpaena*.

Similar exocrine tissue damage was observed by Pallot and Schatzle (14), in carp. But these investigators found no consistent change in alpha or beta cells. Doerr (2), with carp and tench, found no histological changes. Neither investigator included any blood sugar data.

In summary, published reports of the action of alloxan in fish indicate that hyperglycemia may occur in some species and that lesions may be found in alpha cells, beta cells, exocrine tissue, liver, kidney and spleen. To clarify some of the factors concerned in these marked differences among genera, an integrated study of histological and blood sugar responses to alloxan among several fish has been undertaken. The present report describes the consistent and progressive hyperglycemia and beta cell destruction found in the alloxanized catfish.

MATERIALS AND METHODS

Ninety adult catfish, *Ictalurus nebulosus*, (18) (200–600 gm. ± 5 gm.) were placed, in groups of 10, in aquaria provided with aerated, circulating, charcoal-filtered tap water at 10°–15° C. From each tank, 5 fish were randomly selected and injected through the conus arteriosus, as shown in Figure 1, with 10% iced, aqueous alloxan, dissolved less than 5 minutes before use. The remaining 5 fish in each aquarium were injected by the same route with similar volumes of distilled water and served as controls for blood and

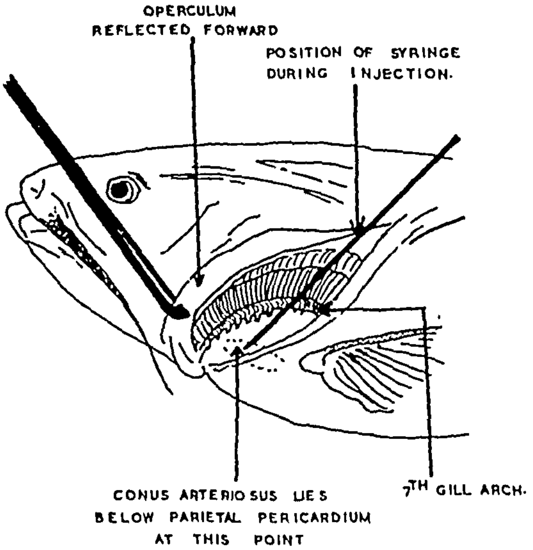


FIG. 1. Injection route.

tissue analysis. The alloxan dose used, 400 mg./kg., produced hyperglycemia in 92% of the experimental fish. At regular intervals, from 3 to 96 hours after treatment, as indicated in Table 1, blood samples were drawn from the conus arteriosus, as suggested by Simpson (19), of the treated and control fish. Immediately after blood sampling, randomly selected control and experimental fish were sacrificed by concussion and islets were removed for histological examination. Blood glucose was measured by a Folin-Malmros micro method, modified for fish blood (10). After fixation in Bouin's fluid, de-

TABLE 1. BLOOD SUGAR* AND BETA CELL† CHANGES AFTER ALLOXAN

Hours after alloxan	Mean* blood glucose, mg.%	Beta granulation†	Other histological features
3	121 ±28	4-II 1-III	None
6	168 ±30	1-II 3-III	Hydropic degeneration in 1; pycnotic nuclei in 2
9	124 ±29	5-III	Pycnotic nuclei and peripheral granulation in 1
12	151 ±25	1-II 4-III	Slight cytoplasmic disintegration; pycnosis in 2
24	169 ±36	1-II 4-III	Cytoplasmic degeneration in 4
48	140 ±12	1-II 1-III 2-IV	pycnotic nuclei in 4, hydropic degeneration in 2
72	157 ±20	2-II 2-III	Pycnosis in 3; Extensive cytoplasmic degeneration
96	150 ±15	2-III 2-IV	Cytoplasmic and nuclear disintegration in 2; many pycnotic nuclei in 2

* Normal mean blood sugar—59 mg.% ±19.

† Arabic numerals in granulation column indicate number of fish observed; Roman numeral indicates degree of granulation, from I (normal) to IV (total degranulation).

hydration in ethyl cellosolve and embedding in 60° Tissuemat, islets were sectioned at 5μ. Sections were stained by Scott's (17) variant of Gomori's aldehyde-fuchsin, with phloxine and fast green FCF counterstains, and by 2 alpha cell methods (1). To avoid errors from possible chance variation in staining, slides were stained in randomized groups, each of which included control and experimental tissues. Islets of 79 fish, 43 control and 36 alloxanized, were classified on an arbitrary scale of beta granulation, from I (normal granulation) to IV (total degranulation). Using another group of 20 fish, we followed blood sugar levels for 14 days, without histological study.

RESULTS AND DISCUSSION

Treated fish differed from control animals in blood sugar level and pancreatic histology. The morphological and blood glucose changes were closely related in time and were progressive through the period studied.

In the intact fish, the mean value for blood glucose was 59 mg.%, with a standard deviation of 19 mg.%. This value was calculated from 237 determinations on 100 normal fish. The total range encountered was 10–120 mg.%. As is shown in Figure 2, extreme values were infrequent.

Alloxanized fish reached blood sugar levels as high as 200 mg.%, (the upper limit of the method) in 179 determinations on 50 animals. The mean value for the experimental group was 121 mg.% 3 hours after treatment, more than twice the mean of controls. In Figure 3 are shown the progress of the hyperglycemia and the comparable control levels. Six hours after treatment, the experimental mean was 170 mg.%, nearly 3 times the normal level. A minor drop, at 9 hours, is not statistically significant. Beyond this period, hyperglycemia persisted for 14 days, when the experiment was ended.

Both in magnitude and variability, the values found resemble those reported by Simpson (19) for catfish, and by others (7, 11, 16) for other fish.

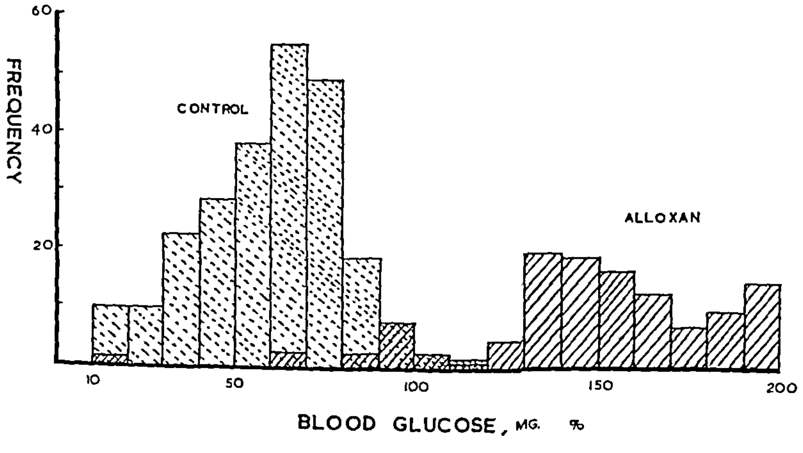


FIG. 2. The distribution of blood sugar levels in control and experimental catfish. The frequency shown at the high end of the blood glucose scale may reflect the 200 mg.% upper limit of the blood glucose method used. Values above 200 mg.% would be read as 200 mg.%.

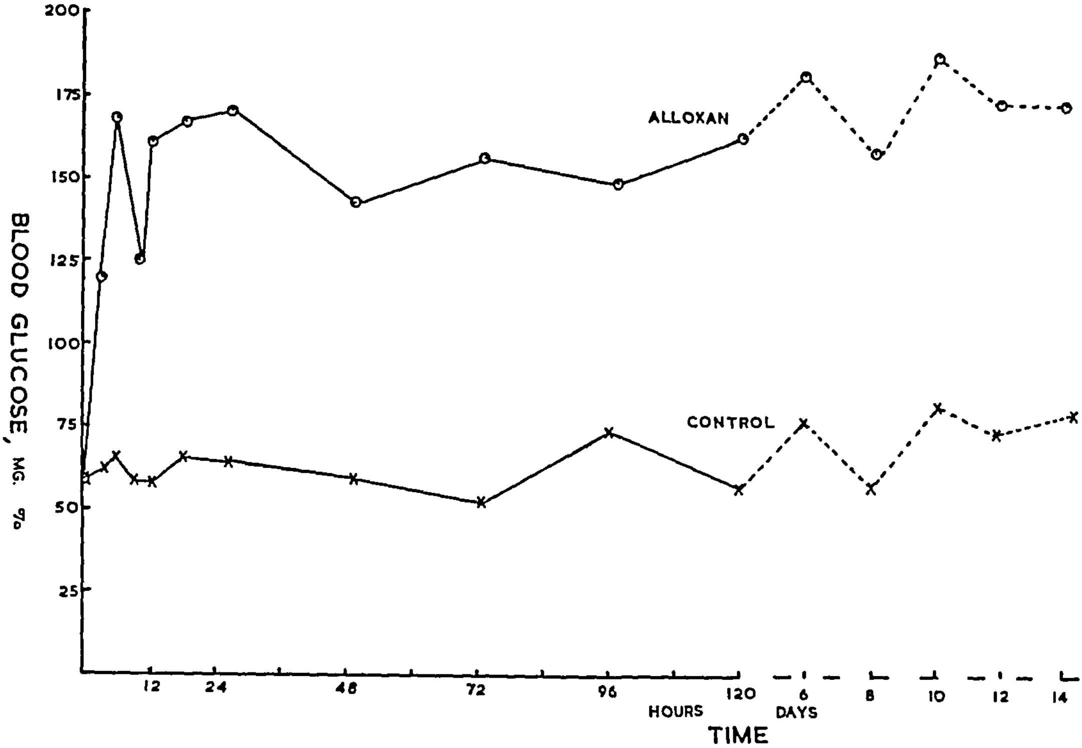
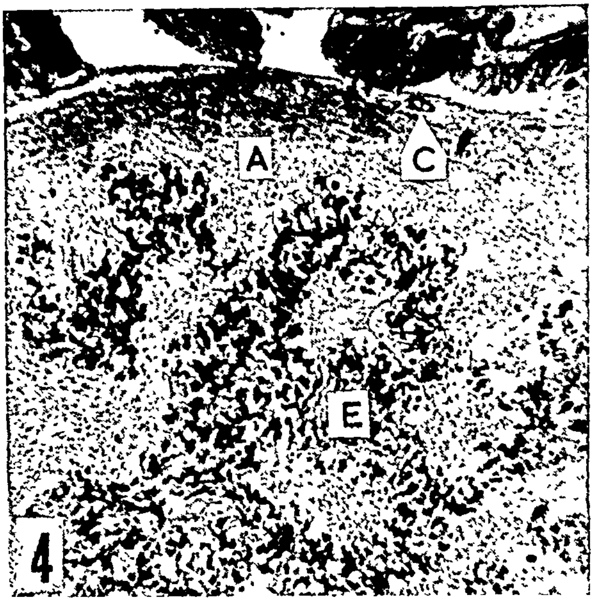


FIG. 3. Effect of alloxan on catfish blood glucose. Broken lines beyond 120 hours indicate reduction of number of fish sampled at each period.

No indication was found of the hypoglycemia observed by Saviano (16), nor was so large a proportion of the treated fish resistant to alloxan. The method used precluded observation of the extreme hyperglycemia found by Saviano in *Scorpaena*, but the frequency distribution plotted in Figure 2 does not suggest that such high values could have been common. The eight-fold increase reported for *Scorpaena* appears unusual for fish or mammals, although comparable to that of steroid-treated fowl. (13).

While the blood sugar changes of the catfish have been found similar to those reported by Saviano, the normal histology and the histological response to alloxan have been found markedly different. The principal islet of the catfish is a nodule, 2–3 mm. in diameter, suspended in the mesentery near the anterior third of the splenic artery. Along the course of this artery are distributed 40–50 minor islets. In islets of both sizes, the richly vascularized endocrine tissue is accompanied by variable, but often extensive, endocrine tissue, as shown in Figure 4. The beta cells form the most common endocrine element, uniformly distributed among alpha cells and a third type of cell, without the localizations described for other fish by Grosso (3) and Saviano (16).

In the normal beta cell, most of the cytoplasm is filled with the characteristic granules, stained intensely with aldehyde-fuchsin, as shown in Figure 5. Striking departures from the normal morphology were found



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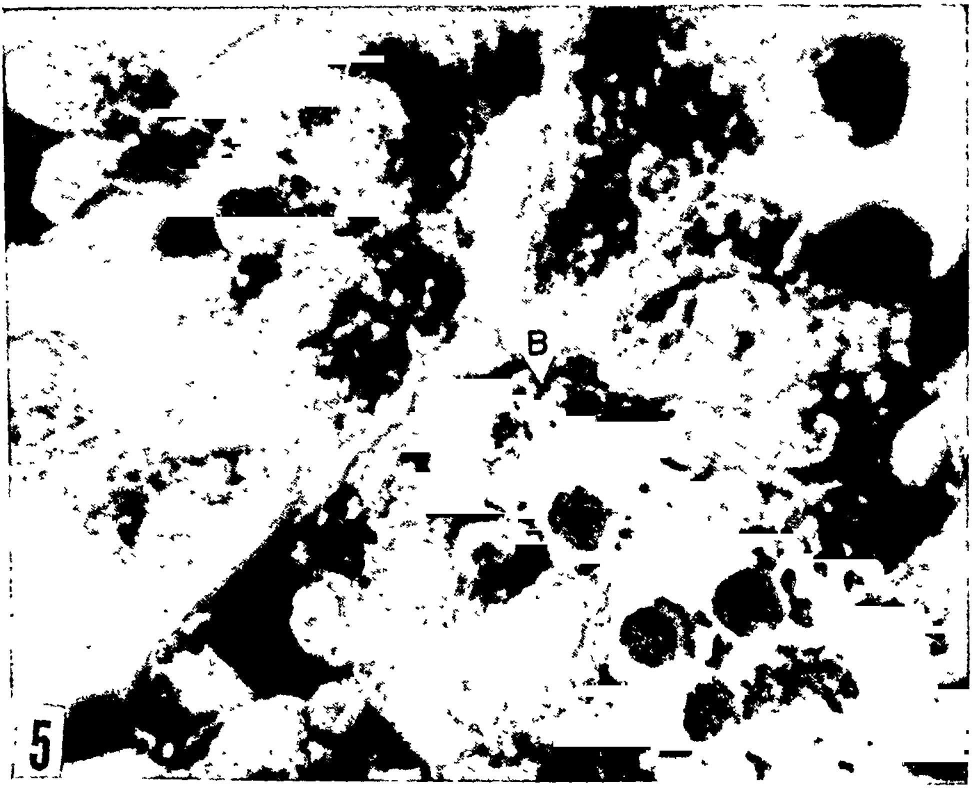

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FIG. 4. Normal catfish pancreas, with exocrine (A), endocrine tissue (E) and connective tissue capsule (C). Aldehyde-fuchsin, ×150.

FIG. 5. Normal endocrine pancreas of catfish with beta granulation (B). Alpha and type 3 cells are intermixed among beta cells. Aldehyde-fuchsin, ×1500.

in alloxanized animals. Degranulation was apparent within 3 hours of treatment and progressed through the period studied, as shown in Figure 5 and Table 1. Six hours after treatment, nuclear and cytoplasmic degeneration were apparent, and became more severe in later samples. The con-

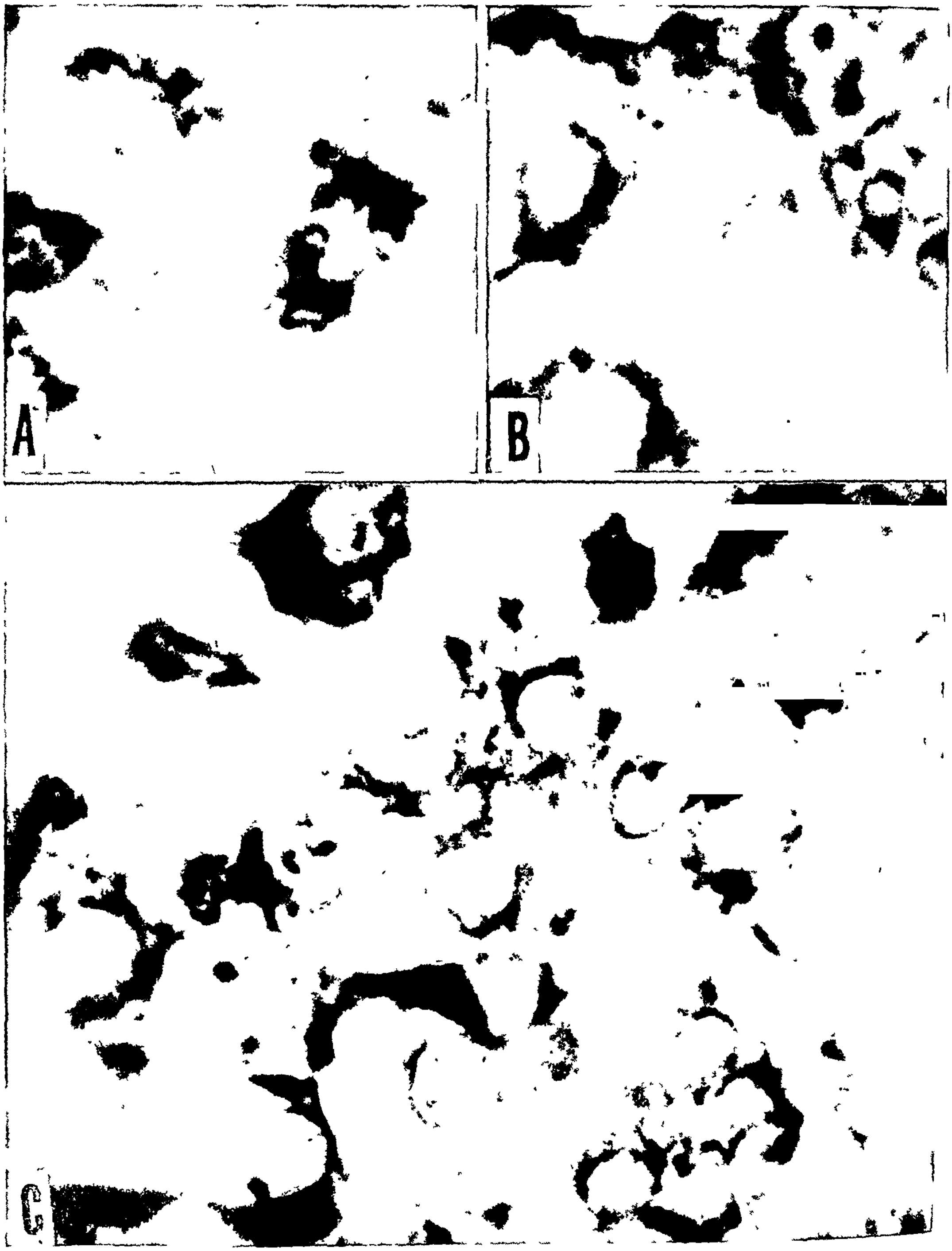

FIG. 6. Pancreatic changes after alloxan. (A) Stage II degranulation. (B) Stage III degranulation. (C) Stage IV degranulation, with nuclear and cytoplasmic damage. Aldehyde-fuchsin, ×1500.

tinuation of this trend through 96 hours suggests an alloxan reaction more prolonged than that of mammals (9, 12). No definite mitotic figures or signs of beta cell recovery were seen, although pairs of cells with small nuclei, suggestive of daughter cells of a recent division, were found in some of the late samples and were never seen in intact animals or early samples.

The tissues studied provided no support for Doerr's explanation (2) of the alloxan resistance of his carp and tench in terms of an extensive supply of immature beta cells available for replacement of those damaged by the drug. The development, in the catfish, of complete degranulation is equally contrary to the observations of Pallot and Schatzle (14), who have described similar reserves of cyprinid fishes. The prominent alpha cell lesions reported by Saviano and the marked zymogen cell damage seen by Saviano and by Pallot and Schatzle were not apparent in the catfish.

The absence of all these features indicates that the histological response of the catfish resembles that of the guppy (3) and of the common laboratory mammals, and differs very markedly from that of other fish, including *Opsanus*,[3] *Scyllium, Motella, Scorpaena,* carp and tench. The clarification of this inherent difference, in this particularly favorable experimental material, should advance understanding of the mechanism of the diabetogenic action of alloxan. The suggestion of Doerr and of Pallot and Schatzle, attributing the alloxan resistance of certain fish to cell reserves and cell transformations, similar to those described in the rat by Hughes (5) and in the guinea pig by Johnson (6), Hausberger and Ramsey (4) and Woerner (20), indicates that comparative studies among fish may be fruitful of understanding of beta cell regeneration and the normal controls of a functional beta cell population.

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[3] Current study of *Opsanus* has demonstrated zymogen cell lesions in this animal, with transitory beta cell destruction and hyperglycemia. Details will be reported shortly.

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BLOOD SUGAR STUDIES IN THE BULLFROG, *RANA CATESBIANA*[1]

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ABSTRACT

Over a period of 2½ years, blood glucose values of 154 normal bullfrogs averaged 13.51 mg.% with 28% showing complete sugar absence. No correlation could be found between blood glucose level and apparent nutritional state of the animal or season of the year. Among factors which induced hyperglycemia were repeated removal of blood samples (without albumin restitution), intra-arterial glucagon >epinephrine, and extracts of bullfrog, angler fish, and dogfish gastrointestinal tissues presumed to contain glucagon. Alloxan, beef STH, and bullfrog pituitary extract failed to raise the blood sugar level significantly. Bullfrogs were somewhat less tolerant of intra-arterially injected monosaccharides than rabbits and responded slowly to exogenous insulin. A brief discussion follows on the relationship of these data to the apparently normal maintenance of an hypoglycemic state in bullfrogs.

IN RECENT studies the author has reported either complete absence of blood glucose (1) or very low sugar values (2) for normal bullfrogs. Wearn and Richards (3) noted absence of blood sugar in several specimens of *Rana pipiens*, attributing their observations to the fact that the frogs had been starved. Apparently little attention was paid to their data since there has never appeared, to my knowledge, any published confirmation or disagreement. Giese (4) reported that activity of laboratory frogs in winter could be improved by glucose injection, but did not indicate that any actual blood sugar values were obtained. Related species, *i.e.*, *Bufo arenarum* (5, 6), *Bufo vulgaris*, *Rana temporaria* and *Rana esculenta* (7), at least when tested by two other standard procedures, regularly show substantial amounts of blood glucose, and no instance of sugar absence has been recorded for any other vertebrate (8).

The discovery of active vertebrates with little or no blood sugar naturally prompted us to look into a variety of factors which might account, separately or in combination, for the anomalous values obtained. The ex-

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periments reported here were designed in the hope of finding some key to the seeming enigma of bullfrog blood glucose.

MATERIALS AND METHODS

Frogs, with a weight range of 80 to 700 gms., were purchased from collectors in Louisiana and Wisconsin except for several specimens found locally in the vicinity of Woods Hole during the summer months. After arrival the animals were stored in granite laundry trays in shallow running water so that their body temperatures were maintained between 16° and 20° C. Because the frogs were used within ten days, no attempt was made to feed them except where specifically mentioned below.

Blood samples were always taken from the conus arteriosus after posteriorly pithing the frog, exposing the pericardial area, and routine heparinization (5 mg./kg.). Since repeated samples from the same frog were often desired, a method was devised whereby a #22 hypodermic needle was left inserted in the right ventral aorta throughout the experiment. All injections were made and all blood removed via this route simply by attaching and detaching various syringe barrels to the stationary needle. Any other method which involved multiple arterial punctures invariably resulted in large blood losses. Some experiments consumed as much as four hours with good circulation maintained throughout; most experimental procedures were completed in less than two hours.

Blood sugar levels were determined according to the method of Nelson (9), except that 0.04N NaOH was substituted for distilled water and $Ba(OH)_2$ in making protein-free filtrates. Duplicate filtrates were made on several occasions with H_2SO_4 and sodium tungstate, but these gave identical sugar values to those made with NaOH and $ZnSO_4$. It should be pointed out that the Nelson technique, because of its greater specificity, gives sugar values 10–15% lower than most other cupric reduction procedures.

EXPERIMENTAL PROCEDURES AND RESULTS

Normal blood sugar values for some 154 animals over the 2½ years of this study gave an average reading of 13.51 mg.%. Distribution of these values over the year (Fig. 1) indicates a tendency for lower glucose readings during the months that northern animals customarily spend in hibernation. Since more than two-thirds of the frogs in this study came from Louisiana where the species is active throughout the year, it is not likely that the preponderance of low glucose values could be attributed entirely to the hibernation state. The general year-around occurrence of instances of glucose absence tends also to indicate against activity as the controlling factor.

A large majority of the frogs was autopsied in an effort to correlate presence of food in the stomach with blood glucose level. No relationship could be discovered, however, since many animals whose stomachs were distended with digesting crayfish had no detectable blood glucose, whereas many frogs with empty stomachs had some of the higher sugar values obtained. A force-feeding experiment was carried out on six frogs by daily rations of beef liver for a period of two weeks (February). Blood glucose on the fifteenth day showed four animals with sugar absence and two with only 5 mg.%. Four animals which received 100 mg. of glucose (5% aqueous solution) intraperitoneally twice daily for four weeks, and twice

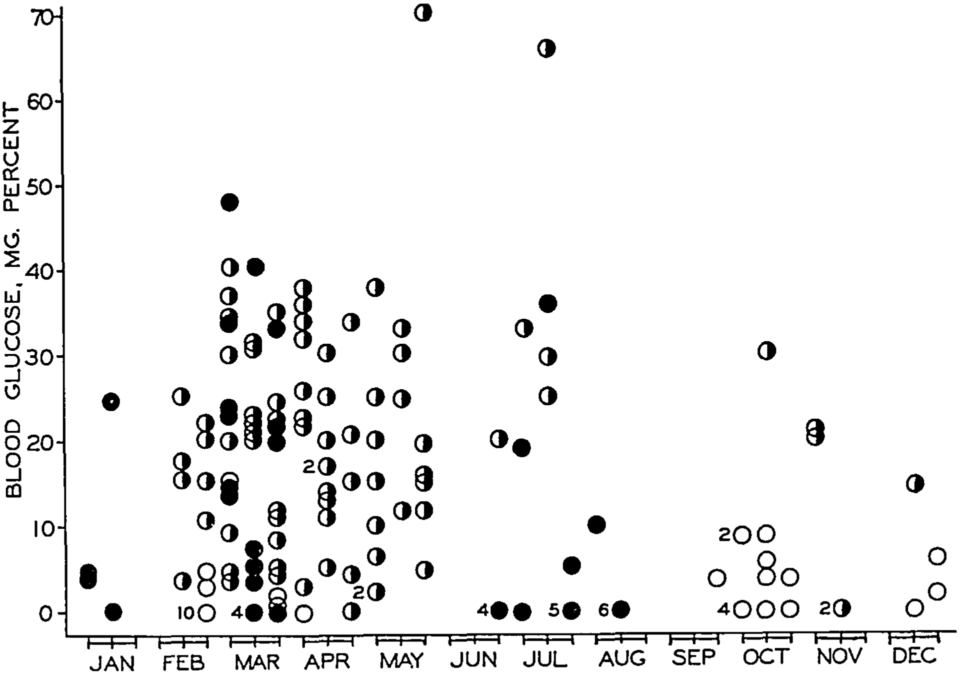

FIG. 1. Blood glucose values for 154 intact bullfrogs as distributed over the year. For convenience months have been divided into thirds, *viz.*, 1st to 10th, 11th to 20th, and 21st to 31st. Open circles, 1956; half-closed circles, 1957; closed circles, 1958.

weekly feedings of beef liver, all gave zero glucose values when blood was taken 16 hours following the last glucose injection.

Among experimental procedures which resulted in some amount of hyperglycemia, a reduction in blood volume (and, consequently, blood pressure) by hemorrhage or repeated removal of blood samples was of primary importance in interpreting early experimental data. Figure 2 shows the progressive hyperglycemic effect of taking numerous blood samples and the correction of this difficulty by injection of an equal volume of 3% human blood albumin in Ringer's solution following removal of each blood sample. Fortunately, the hypergylcemic effect of reduced blood volume was discovered early in experimentation so that routine replacement of blood with albumin was an integral part of all experiments reported below. It is assumed without any supporting evidence that hyperglycemia following a drop in blood volume and blood pressure comes about reflexly, as in mammals, through the endogenous release of epinephrine or through general sympathetic stimulation.

Response of bullfrogs to glucagon injection is dramatic, but is probably more closely related to some uncontrollable factor such as the indefinable nutritional state of the animal than to the dosage of hormone administered (Table 1). The sharpest rise in blood sugar level occurs within the

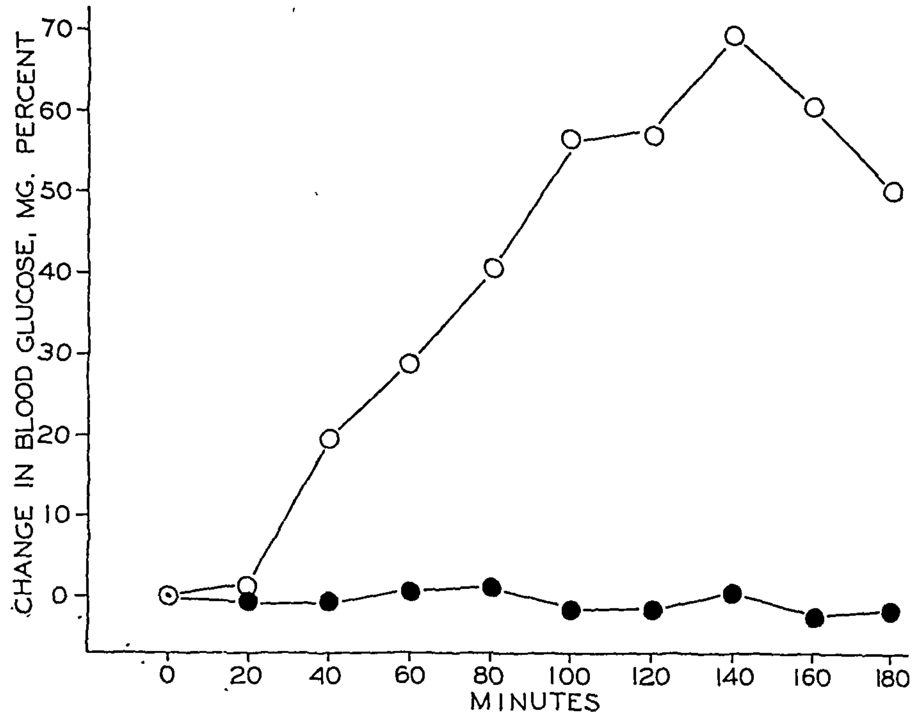

FIG. 2. Spontaneous development of hyperglycemia due to taking of repeated blood samples every 20 min. (open circles). Maintenance of nearly constant blood glucose level when blood as removed is replaced at once with equal aliquots of 3% blood albumin in Ringer's solution (closed circles). March.

first 20 minutes after glucagon injection, the peak response ordinarily occurring, with amorphous glucagon, within the first 40 minutes. Crystalline glucagon seemed to produce somewhat lower levels of hyperglycemia during the first 20 minutes, but the effect was a progressive one up to at least 90 minutes after injection. In contrast to these data, epinephrine injection (10) raised the blood sugar level by much smaller amounts (av. 32 mg.%, max. 45 mg.%).

In view of the difference in degree of response to epinephrine and glucagon, it seemed interesting to investigate the effects of a second dosage of either epinephrine or glucagon after an original administration of one of the hormones had had ample opportunity to act. Results (Table 2) showed that even though a moderate dosage of glucagon had been allowed to exert its effect, a second dosage produced a still greater degree of hyperglycemia. If glucagon was administered 40 minutes following a dosage of epinephrine, a second, more marked, wave of hyperglycemia occurred. If, on the other hand, epinephrine was injected 40 minutes after administration of glucagon, no noticeable hyperglycemic response was produced by epinephrine.

Alloxanization (up to 800 mg./kg. intraperitoneally) failed to induce any hyperglycemia nor any detectable islet damage upon microscopic examination of Gomori-stained tissue. Animals seemed to tolerate the

TABLE 1. RESPONSE OF BULLFROGS TO INTRA-ARTERIAL INJECTION OF GLUCAGON*

Date	Glucagon	Dosage, μg./kg.	Blood sugar, mg.% at				
			0 min. (initial)	20 min.	40 min.	60 min.	90 min.
3-19	amorph.	10	0	4	0		2
7-15		10	25	24	22	27	
4- 2		25	0	112			
5-20		30	25	94	88	107	141
4- 3		40	36	98	103		138
5-17		50	12	47	77	62	
5-17		100	30	81	88	79	106
5- 5		150	10	96	125	107	121
5-21		200	15	60	42		44
5-24		200	70	170	209	252	206
1-15	cryst.	10	0	0	5	0	4
1-15		25	25	83	135	141	
1- 9		50	4	54	63	75	
7-15		100	36	65	69	86	93
7-10		200	19	33	37	41	50

* Dr. W. R. Kirtley, Lilly Laboratories, gratefully supplied the glucagon used. Amorphous glucagon was from batch #258-234B-40 and crystalline glucagon from lot #258-234B-54-2. Both were used as a 0.02% solution in glycine buffer, pH 9.4, stored in a refrigerator.

alloxan well, as there was no mortality among six animals for ten days following injection of the drug. Attempts to induce the diabetic state by total or subtotal pancreatectomy were unsuccessful, for no frog survived longer than an hour or two following the operation. A large portion of the frog pancreas surrounds the common bile duct and a portion of the the hepatic portal vein, making even subtotal pancreatectomy extremely difficult.

TABLE 2. RESPONSE OF BULLFROGS TO REPEATED DOSAGES OF HYPERGLYCEMIC AGENTS

Date	Original agent inj. at 0 min.	Blood sugar, mg.% at			Second agent inj. at 40 min.	Blood sugar at	
		0 min. (initial)	20 min.	40 min.		60 min.	80 min.
4-26	AG*	34	74	87	AG	116	201
5- 1	AG	25	45	40	AG	75	134
5- 3	AG	3	59	50	AG	71	100
4-29	AdCl†	0	5	19	AG	73	127
5- 8	AdCl	15	20	30	AG	112	118
5-10	AG	3	74	85	AdCl	70	80
5-10	AG	38	92	102	AdCl	83	82

* Amorphous glucagon, 100 μg./kg.
† Adrenalin chloride, Parke, Davis Co., 100 μg./kg.

Administration of beef STH[3] also failed to induce hyperglycemia both when given in twice-daily intraperitoneal injections (2.5 mg. each) for two days prior to pithing and when given, like glucagon, intra-arterially (5 mg.) following pithing. Injection of crude bullfrog anterior lobe extract (Ringer) induced a very small (10–15 mg.%) rise in blood sugar 40 minutes after injection. Nothing is known of the STH content, if any, of such a preparation. Four bullfrogs, hypophysectomized from 3 to 7 days previously, had no detectable blood glucose.

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[3] Supplied by Dr. Sanford L. Steelman, Armour Laboratories.

Extracts of gastro-intestinal tissues of the bullfrog, prepared according to the method of Sutherland and de Duve (11), showed the presence of some moderately potent hyperglycemic agent presumed to be glucagon in pancreas > gastric mucosa > duodenum. These preparations were incubated for 4 hours in 0.1% cysteine to destroy any possible traces of epinephrine or its analogues. Blood glucose rises of from 20 to 65 mg.% were achieved within 40 minutes after intra-arterial injection of these extracts, dosages (0.5 to 1.0 cc. in volume) being equivalent to 0.25 to 1.0 gm. of fresh tissue. Injection of these same dosages into rabbits failed to induce any hyperglycemia, suggesting that amphibian glucagon, if present in these extracts, differs significantly from mammalian glucagon.

Injection of 0.5 cc. extract (representing 0.4 gm. fresh tissue) of the principal islet of the angler, *Lophius piscatorius*, induced a 41 mg.% rise in frog blood glucose in 40 minutes. There was a 46 mg.% rise in blood sugar of a bullfrog 40 minutes after injection of 1.0 cc. extract (1 gm. fresh tissue) of dogfish, *Mustelus canis*, pancreas. Extracts of bullfrog pectoralis muscle prepared in the same way as the above tissues, induced some hyperglycemia (as much as 50 mg.% in 40 minutes) before incubation with cysteine, but this potency was completely lost after cysteine treatment.

Sugar tolerance tests on bullfrogs showed them to compare favorably with mammals in ability to bind injected monosaccharides and even maltose. Injection of Evans blue and subsequent colorimetric analysis showed the blood volume to be 6.5 to 7% of body weight. On this basis bullfrogs convert 52% of glucose (4 cases), 51% of galactose (3 cases), 49% of fructose (4 cases), 45% of arabinose (2 cases), and 38% of mannose and maltose (2 cases each) within five minutes after intra-arterial injection, each sugar dosage having been calculated to raise the blood sugar level by 100 mg.%. The relatively slow hypoglycemic action of insulin is well demonstrated when the hormone is used to modify the tolerance curves of glucose and fructose (Table 3).

Simultaneous injection of monosaccharide along with crystalline glucagon gave interesting results (Table 3) in that injected sugar seemed to inhibit the continuing hyperglycemic action of glucagon. Equally interesting is the observation that glucagon injection did not inhibit the usual amount of glycogenesis which normally followed injection of these sugars.

DISCUSSION

This paper has not attempted to deal with perhaps the most intriguing problem presented by bullfrogs, *i.e.*, how can such a vertebrate manage to survive, or indeed show appreciable activity, with blood glucose unavail-

TABLE 3. EFFECT OF INSULIN AND GLUCAGON ON THE MONOSACCHARIDE TOLERANCE CURVES OF BULLFROGS

Date	Sugar*	Hormone†	Blood sugar, mg.% at				
			0 min. (initial)	5 min.	30 min.	60 min.	90 min.
3- 6	Fructose	None	48	84	94	79	74
3- 8	Glucose	None	33	80	86	72	70
3- 5	Fructose	4 u. Insulin	34	107	90	63	48
3-25	Fructose	8 u. Insulin	0	54	27	22	38
2-22	Glucose	4 u. Insulin	20	53	43	36	26
3- 1	Glucose	4 u. Insulin	9	68	44	32	25
3-20	Glucose	8 u. Insulin	0	59	44	37	33
3-26	Fructose	Glucagon	1	47	26	38	38
3-26	Glucose	Glucagon	5	55	32	35	35

* Sugar dosages were calculated to raise the blood sugar level by 100 mg.% on the basis of body weight and were administered in 0.1% saline solution immediately after initial blood sample.

† Insulin or glucagon was given intra-arterially 10 minutes after sugar. Insulin was Iletin Lilly (40 units per cc.) and glucagon was crystalline in a dosage of 50 μg./kg.

able or in extremely short supply? The fact that more than 25% of animals tested showed absence of glucose indicates that the whole species must be subject at one time or another to lack of blood carbohydrate. It may be convenient to say that frog tissues have become accustomed to doing without ready sugar, but to make this statement in no way answers the question of how cells have modified their metabolism to meet this unusual circumstance. The answer to this phase of the problem must be provided by biochemical studies at the level of blood-tissue interrelationships. Any suggestions presented at this time would be merely guesses for lack of information; nevertheless, the subject is a tempting one for much speculation.

As to explanations for the maintenance of blood glucose at such low levels, the results presented in this communication have not appeared to answer that phase of the problem, either. Except possibly for its negative response to STH and the minimal response to pituitary extracts, the frog seems to react to experimental procedures in virtually the same manner as higher vertebrates which customarily maintain considerably higher blood glucose levels. Frogs were even less tolerant of injected sugar than rabbits, which in our laboratory converted an average of 75% of glucose within five minutes after intravenous injection, as opposed to the frog's 50% or so. This response indicates that frogs perhaps have a relatively lower level of circulating insulin, and therefore have the capacity for maintaining a higher level of blood glucose than do rabbits. It may also be inferred from the results of monosaccharide injection that the appearance of sugar in the blood stream does not induce a surge of endogenous insulin secretion which, if prolonged, might account for the maintenance of low sugar levels. The slow response to exogenous insulin indicates a relative insensitivity of frog tissues to this hormone, and would tend, if anything, to allow hyperglycemia rather than the severe hypoglycemic condition encountered.

Responses to glucagon put the frog in the mammalian category, apart from the only other amphibian whose reaction to glucagon has been tested. Miller and Wurster (12) found that glucagon did not raise blood sugar in *Taricha torosa*. This salamander also has normally low blood glucose (15 to 20 mg.%), but not glucose absence, and does not appear to have alpha cells within its pancreatic islets. Bullfrogs definitely have islet cells which give the customary reactions of alpha cells when stained with Gomori's chrome hematoxylin and phloxin or with aldehyde fuchsin and trichrome (author's unpublished study).

The failure of bullfrogs to respond to beef STH and the weak response to a frog pituitary extract should have further investigation. It would also be helpful, indeed, to know the response to STH as isolated from fish, or better still, frog, pituitary glands, for it is very possible that the frog is insensitive to this pituitary factor as derived from a mammal. If STH from lower forms failed to induce hyperglycemia in frogs, it is entirely possible that an explanation for the widespread hypoglycemia could lie in that relationship. Even though glucagon appears to be present in frog gastro-intestinal tissues, it may not function in the absence of a factor to effect its release. In that event, levels of insulin, however high or low, would then be unopposed and could account for the normally hypoglycemic state.

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STUDIES ON THE MECHANISM OF HYPOTHALAMIC CONTROL OF THYROTROPIN SECRETION: EFFECT OF THYROXINE INJECTION INTO THE HYPOTHALAMUS OR THE PITUITARY ON THYROID HORMONE RELEASE[1]

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ABSTRACT

In an attempt to ascertain whether or not thyroxine can change hypothalamic as well as pituitary activity in regulating thyrotropin secretion, microinjections of thyroxine were made directly into a localized area of the hypothalamus or into the anterior pituitary. Determination of thyroid secretion was utilized in evaluating possible changes in thyrotropin secretion. Inhibition of thyrotropin secretion was produced by injection of thyroxine into either the "thyrotropin area" of the hypothalamus or into the anterior pituitary. Injection into the subarachnoid space or into other areas of the hypothalamus was without effect. Hypothalamic injections had a latent period of six to nine hours before thyrotropin secretion was inhibited, whereas the inhibition was immediate after pituitary injections. The duration of inhibition was approximately 20 hours in either location. It is suggested that two separate mechanisms might control thyrotropin secretion, a slower one acting through the central nervous system and a direct one in the anterior pituitary itself.

MANY workers have studied the effect of hypothalamic lesions, pituitary stalk section or electrical stimulation of the hypothalamus on thyroid function. Recent studies by Greer (1, 2) have demonstrated that destruction of the anterior hypothalamus of the rat prevents the thyroid hypertrophy which normally follows propylthiouracil treatment. This has been confirmed by Bogdanove and Halmi (3) and others (4, 5). It thus seems well established that the hypothalamus plays an important role in the control of thyrotropin secretion by the pituitary.

It has been postulated that thyroid hormone controls thyrotropin secretion by altering the activity of the hypothalamus and thereby the pituitary (6). Such a hypothesis is attractive, but there is no actual proof of this mechanism and the primary role of the hypothalamus is still uncertain.

In the present study, in an attempt to ascertain whether or not thyroxine can change hypothalamic as well as pituitary activity in regulating thyrotropin secretion, microinjections of thyroxine were made directly into a localized area of the hypothalamus or into the anterior pituitary. Deter-

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mination of thyroid secretion was utilized in evaluating possible changes in thyrotropin secretion.

MATERIALS AND METHODS

Forty seven adult male Holtzman rats, weighing 212 to 344 gm., were used. Animals were fed a Remington low iodine diet[2] for at least 7 days in order to get a high uptake of I^{131} by the thyroid gland. Animals used in the hypothalamic injection experiments were divided into three groups: Posterior, Anterior and Pre-optic. The number of experimental and control animals in each group is shown in Table 1.

TABLE 1. SUMMARY OF THE RESULTS OF SALINE AND THYROXINE INJECTION INTO THE HYPOTHALMUS OR PITUITARY ON THYROIDAL SECRETION

	Saline controls (No. of animals)	*Thyroxine injection*			*Inhibition*	
		No. of animals	Exhibiting depressed thyroid secretion		Onset aft. T4 inj. (hrs.)	Duration (hrs.)
			(Number)	(Per cent)		
Hypothalamus Group						
I. Post.	2	3	1	33	12	16
II. Ant.	7	18	16	89	8.5±1.2[1]	20.6±1.1
III. Pre. Op.	—	2	0	0	—	—
Pituitary Group						
Pituitary	3	4	4	100	<0.5	20.6±6
Subarachnoid space	2	2	0	0	—	—

[1] Mean and S.E.

Number 27 hypodermic needles, 0.3 mm. in external diameter, were inserted in the midline with a stereotaxic instrument (11) employing the following parameters for each group: Posterior, 4.5 mm. anterior to the ear bar, 8.5 mm. deep (from the top of the skull); Anterior, 6.5–7mm. anterior, 8–8.5 mm. deep; Pre-optic, 8 mm. anterior, 8 mm. deep. In the Pituitary group, the needles were inserted in the anterior lobe. After insertion, the needle was fixed to the skull by four watch case screws (Fig. 1).

l-Thyroxine in propylene glycol[3] was diluted with physiological saline to a concentration of 0.25 μg. thyroxine /0.02 ml. saline.

Twenty-four hours after the intraperitoneal administration of 20 μc. of I^{131}, 30 mg. of propylthiouracil in 10% gum acacia solution was given subcutaneously daily at rotated sites to block the reaccumulation in the thyroid of I^{131} liberated from degradation of endogenously labeled thyroxine. Counts over the thyroid region were made five times daily under light ether anesthesia by means of an externally placed scintillation counter and each point was expressed as a per cent of initial (24 hour) thyroid uptake and plotted on semi-log paper.

After obtaining a straight line of more than five individual points, 0.25 μg. *l*-thyroxine contained in 0.02 ml. saline was injected into the hypothalamus, pituitary or subarachnoid space through the needles previously fixed to the skull. In the control animals of each group, 0.02 ml. saline were injected in the same way. The "dead space" of each needle was taken into account when calculating the volume injected. This averaged 0.02 ml.

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[2] Iodine Deficient Test Diet supplied by General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Kindly supplied by Dr. A. E. Heming, Smith, Kline & French Laboratories.

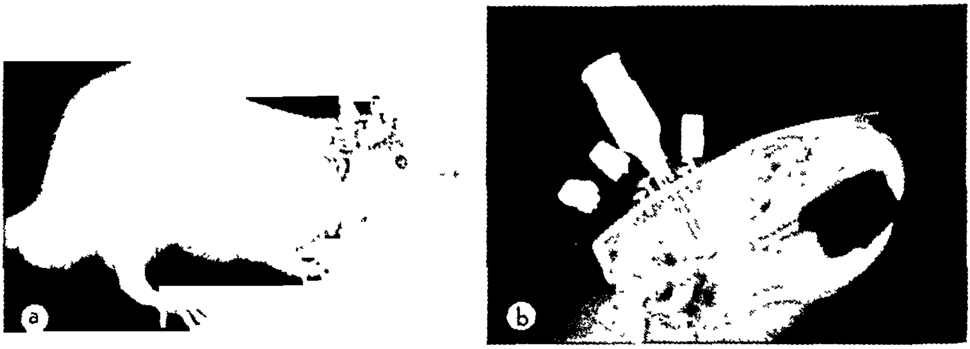

FIG. 1. Needle inserted into the anterior hypothalamus of a rat. (a) Photograph of rat with needle in place; (b) Radiograph of skull with fixed needle.

In the animals in which a possible stress effect of the microinjection was studied, 0.02 ml. physiological saline was injected into the anterior hypothalamus or the pituitary one hour after I^{131} injection, and the 24 hour uptake by the thyroid gland was measured by *in vivo* counting.

The exact position of the needles, cytological reaction around the needles, lesions due to the needles and possible infection were studied in 21 of the 29 thyroxine-injected animals in serial histological sections cut at 10 μ and stained with hematoxylin-eosin.

RESULTS

Thyroid hormone release in the non-operated control group was measured for about 100 hours. The plots obtained in four animals were exponential throughout the period of the experiment, although several points were rather irregular.

Before starting the microinjection experiment using thyroxine, the effect of systemic injection of thyroxine (0.25 μg.) and the possible non-specific effect of microinjection into the hypothalamus and the pituitary were studied. None of the animals which received a systemic injection of 0.25 μg. thyroxine showed a change in the release slope (Fig. 2). However, complete plateauing of the slope or slowing of the rate persisted for about 19 hours in animals that received systemic injections of 5 μg. thyroxine. Onset of inhibition seemed to be within four hours in all affected animals.

The effect of microinjection of saline into the hypothalamus or the pituitary on thyroidal uptake of I^{131} is shown in Fig. 3. The 24-hour uptake was 20.8%, 18.7% and 19.9% respectively in the Anterior (hypothalamic) injection group, the Pituitary group and the untreated control group. Statistically there was no significant difference between them.

1. *Effect of Hypothalamic Injection of Thyroxine on Thyroid Hormone Secretion*

In the Posterior group, a change of slope was observed in one of three experimental animals. Twelve hours after injection, thyroidal secretion

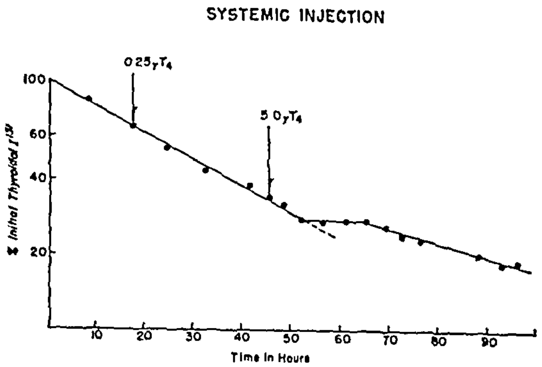

FIG. 2. The effect of systemic injection of 0.25 μg. l-thyroxine and subsequent systemic injection of 5 μg. l-thyroxine on thyroid hormone secretion. After 0.25 μg. no change was produced, but after 5 μg. transient complete inhibition occurred.

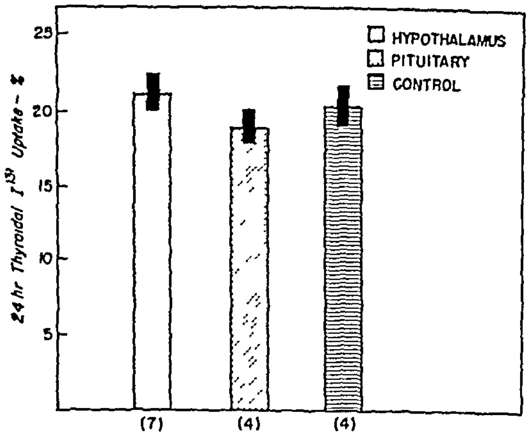

FIG. 3. Effect of saline injection into the anterior hypothalamus and the pituitary on thyroidal radioiodine uptake. 0.02 ml. saline was injected one hour after I^{131} injection in the hypothalamus and pituitary group. Number of animals is indicated in parentheses.

was completely inhibited. This inhibition persisted for 16 hours.

In the Anterior group, a change in rate occurred in 16 of 18 animals. The time of onset varied from animal to animal, but most striking was the fact that inhibition occurred 2 to 16 hours, usually 6 to 9 hours, after thyroxine injection (average 8.5 hours), while in only two animals did a change in slope occur immediately. The duration of inhibition also varied from animals to animal, but there was less fluctuation than with time of onset. The average length of inhibition was 20.6 hours. In most of the animals, complete flattening occurred (Fig. 4), but in some animals only a change of slope was observed (Fig. 5). It was difficult to detect any relationship between the onset of change, degree of change, or its duration.

In the Pre-optic group, none of the experimental animals showed a decreased thyroidal secretion after thyroxine injection. In no instance did injection of saline change the rate of loss of I^{131} from the thyroid.

Thyroxine was injected twice in five animals, three of the Anterior group and two of the Posterior group. A change in thyroidal secretion rate occurred both times in the Anterior animals. The onset of change and the duration were in good agreement in all three rats (Fig. 6). In the Posterior animals no change was produced by either injection of thyroxine (Fig. 7).

II. *Effect of Injection of Thyroxine into the Pituitary on Thyroid Hormone Secretion*

In the Pituitary group, it was observed that a change of slope occurred immediately after thyroxine injection in all four animals, whereas there

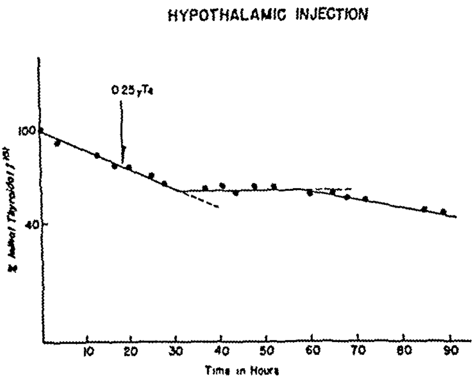

FIG. 4. Ten hours after injection of 0.25 μg. *l*-thyroxine into the anterior hypothalamus, complete inhibition of thyroidal secretion occurred and lasted approximately 30 hours.

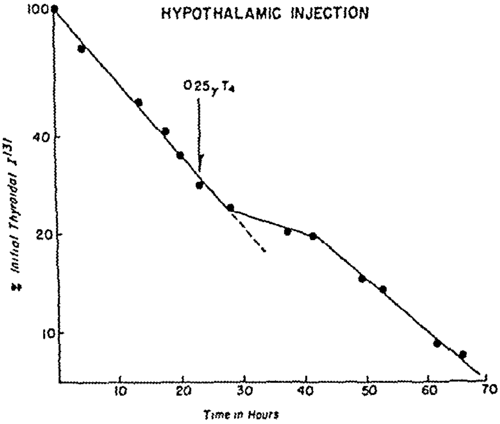

FIG. 5. Five hours after injection of 0.25 μg. *l*-thyroxine into the anterior hypothalamus, a change of slope occurred which lasted about 14 hours.

was no effect from saline injection into the pituitary nor from thyroxine introduced into the subarachnoidal space under the pituitary. In two of four experimental animals the slope became completely flat (Fig. 8), while in another two animals only a slowing of the rate occurred (Fig. 9). Duration of the inhibition of thyroidal secretion varied from animal to animal as in the hypothalamic experiment, but the average was 20.6 hours, being very close to that produced by hypothalamic injection of thyroxine.

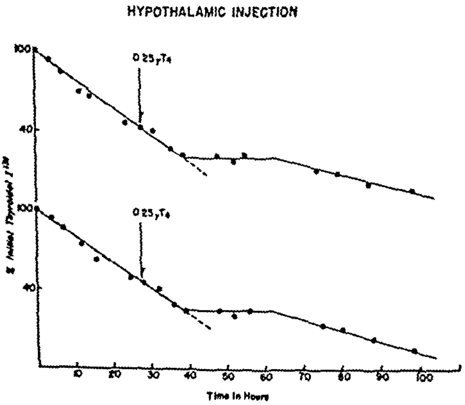

FIG. 6. Repeated experiments in the same animal of the anterior hypothalamus group. The first experiment is above and the second below. In both experiments, inhibition occurred about ten hours after thyroxine injection and lasted for 14 hours.

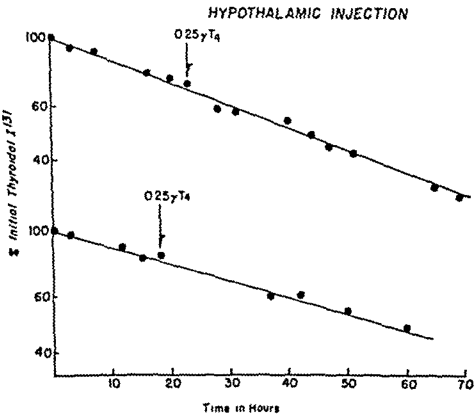

FIG. 7. Repeated experiments in the same animal of the posterior hypothalamus group. In both experiments, no change in thyroidal secretion was produced after thyroxine injection.

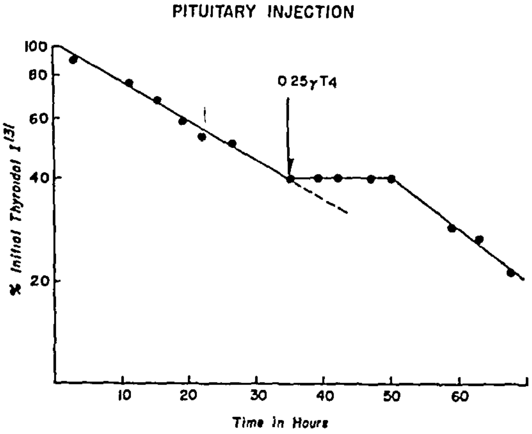

FIG. 8. Soon after the injection of T_4 into the pituitary, complete inhibition lasting 24 hours occurred.

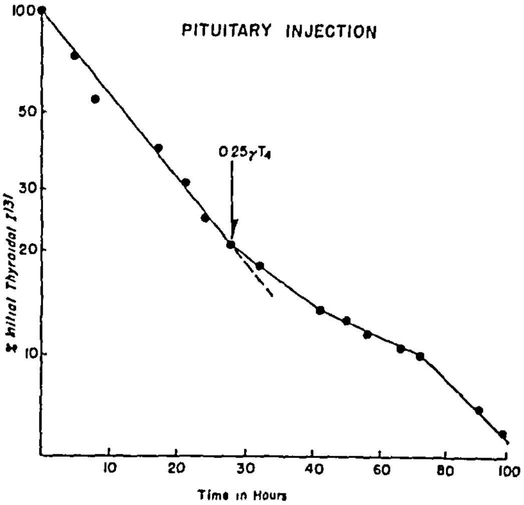

FIG. 9. In this animal, following thyroxine injection into the anterior pituitary, the slope changed but did not completely flatten. This partial inhibition lasted for about 25 hours.

III. *Histological Examinations*

The position where the needles were inserted is shown in Fig. 10. In the Posterior group, the needle in the animal which gave a positive result was situated nearer to the so-called "thyrotropin area" than in the animals which had no change in thyroidal secretion after thyroxine injection.

In the Anterior group, in all animals studied the needles were situated in the thyrotropin area. It was of interest that, in the two animals which gave a negative result, the needles penetrated slightly through the base of the hypothalamus, indicating that the injected thyroxine may not have entered the hypothalamus.

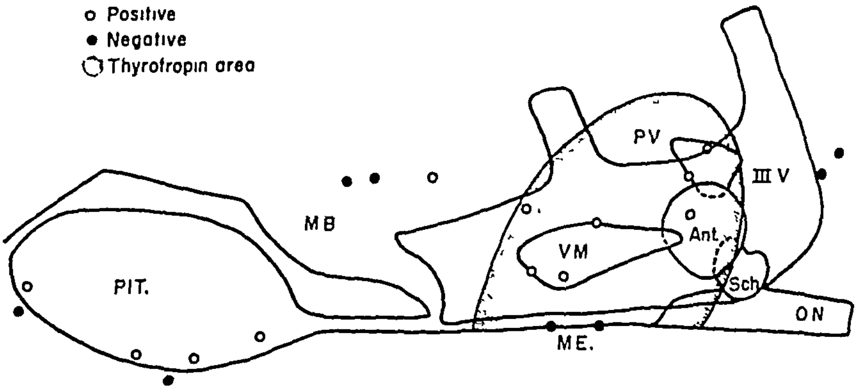

FIG. 10. Diagram of the position of the inserted needles. Solid circles indicate a negative effect and open circles a positive effect. The thyrotropin area is surrounded by a stippled border. P.V. = paraventricular nucleus, III V. = third ventricle, Ant. = anterior hypothalamic area, Sch. N. = suprachiasmatic nucleus, V.M. = ventromedian nucleus, M.E. = median eminence, M.B. = mamillary body, PIT = pituitary.

In the Pre-optic group, the needles were located anterior to the thyrotropin area in both animals. In the Pituitary group, the needles were in the anterior pituitary in all animals; they were just below the pituitary in the Subarachnoidal group.

There was no definite correlation of the anatomical position with the degree or duration of inhibition. Some cytological reaction around the needles was observed, but it was very slight. No lesions or infection due to the needle insertion were found.

DISCUSSION

Previous studies have shown that only lesions in the so called "thyrotropin area" of the hypothalamus, in the midline between the paraventricular nucleus and the median eminence, consistently result in diminution of thyrotropin secretion by the pituitary (7). It has also been shown that the neocortex is not essential to thyrotropin release in the rat (8). Such studies suggest that the hypothalamus may be a primary center controlling thyrotropin secretion from the pituitary although the possibility remains that the hypothalamus is merely an intermediate area transmitting impulses from higher centers to the pituitary.

Von Euler and Holmgren (9) reported that microinjection of systemically ineffective amounts of thyroxine directly into the anterior pituitary would cause a decrease in the release of radioiodine from the rabbit thyroid but that the same amount of thyroxine was without effect when injected into the median eminence or other parts of the hypothalamus. They concluded that the inhibitory effect of thyroxine on thyrotropin secretion was produced only by direct action on the anterior pituitary (9, 10).

In the present study, however, inhibition of thyrotropin secretion was produced by injection of thyroxine into either the "thyrotropin area" of the hypothalamus or into the anterior pituitary. As previously shown by Von Euler and Holmgren (9), injection into the subarachnoid space was ineffective. Thyroxine placed in other areas of the hypothalamus was also without effect. The two animals injected into the "thyrotropin area" in which no inhibition was produced apparently had leakage of the thyroxine into the subarachnoid space, as indicated by the histologic sections of the hypothalamus.

No effect from the injection of similar amounts of saline on either thyroidal secretion or radioiodine uptake was seen.

The data presented here clearly indicate that both hypothalamic and pituitary injection of systemically ineffective amounts of thyroxine can inhibit thyrotropin secretion. Of particular interest is the observation that hypothalamic injection has a latent period of six to nine hours before thyrotropin secretion is inhibited whereas the inhibition is immediate after pituitary injection. The duration of inhibition is approximately the same when thyroxine is injected into either location, being about 20 hours.

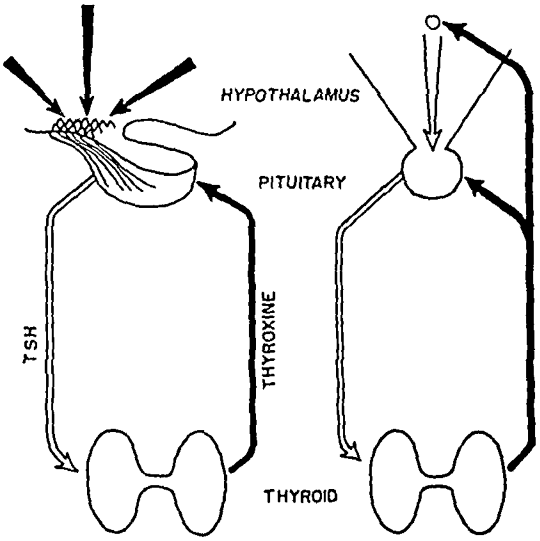

FIG. 11. Diagram of alternate hypotheses to explain the observed inhibitory effect on thyrotropin secretion by hypothalamic thyroxine injection. (1) On the left is shown the possibility of diffusion of injected thyroxine to the origin of the hypophyseal portal vessels and its transport to the anterior pituitary where the inhibitory effect is exerted. (2) On the right is shown a possible negative feed-back mechanism in both the hypothalamo-pituitary-thyroid system and the pituitary-thyroid system.

Two possible explanations of the data presented above can be made (Fig. 11): (1) Thyroxine injected into the anterior hypothalamus takes approximately 8.5 hours to diffuse to the hypophyseal portal vessels and be carried to the anterior pituitary where it exerts an inhibitory effect. (2) Direct inhibition of a primary hypothalamic "thyrotropin center" is produced by thyroxine injection into the anterior hypothalamus. Cells in this area are sensitive to alterations in circulating thyroid hormone concentration but there is a latent period of about 8.5 hours before thyrotropin release by the pituitary is eventually affected by this neural or neurohumoral mechanism.

It is not possible to decide which, if either, of these hypotheses may be correct at present, but it seems unlikely that it would require several hours for thyroxine to diffuse to the anterior pituitary. It seems more reasonable that two separate mechanisms can control thyrotropin secretion, a slower one acting through the central nervous system and a direct one in the anterior pituitary itself.

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RAPID DISAPPEARANCE OF PROGESTERONE FROM OILY SOLUTIONS INJECTED INTRAPERITONEALLY IN MICE[1]

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ABSTRACT

Seventy-five to 22,500 μg. of progesterone dissolved in 1.5 ml. sesame oil were injected intraperitoneally into ovariectomized adult CHI mice. They were exsanguinated two and one-half minutes to eight hours later, and the gestagen levels in the plasma and in the oil recovered from the peritoneal cavities were determined by bio-assay. Concentrations in the plasma were found to have risen quickly and to have declined within an hour. Concentrations in the recovered oil fell to 60 μg. or less within 10 minutes and to about 12 μg. in 30 minutes, regardless of the original concentration of the injected solution.

THIS experiment was undertaken primarily to determine the rate at which progesterone moves out of an oily solution after its intraperitoneal injection. Resulting concentrations of gestagen in the plasma were also investigated. Some of the results have already been briefly reported (1).

PROCEDURE

Young adult CHI mice were bilaterally ovariectomized. Eight days after the operation each mouse received an intraperitoneal injection of sesame oil. Five control mice were injected with 1.5 ml. oil only. Eighty-nine other mice were injected with various quantities of progesterone dissolved in 1.5 ml. oil, as follows: 75 μg., 18 mice; 1,500 μg., 47 mice; 2,250 μg., 6 mice; 7,500 μg., 6 mice; 15,000 μg., 4 mice; 22,500 μg., 8 mice. At a selected interval after the injection each mouse was anesthetized with ether, and the carotid sheath was rapidly exposed and divided. The blood was collected in a 1 ml. centrifuge tube containing 0.05 ml. of 20% sodium citrate solution as an anti-coagulant. The abdomen was opened, and as much injected oil as possible (0.3–0.8 of the original 1.5 ml.) was aspirated. Recovery of the oil sample took an average of about ten minutes. The sample was centrifuged to separate the oil from the small amount (insufficient for assay) of peritoneal fluid also present.

The blood was centrifuged promptly, and a measured volume of plasma was added, drop by drop, with stirring to several volumes of cold (10° C) acetone. The resulting precipitate was separated by centrifugation and was washed three times with equal volumes of acetone and ether. The original supernatant and the washings were pooled, and to them was added a measured volume of sesame oil. The acetone and ether were re-

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moved by distillation. The residue of oil and of water from the plasma was centrifuged to separate the two phases, and the oil was bio-assayed for the acetone- and ether-soluble "free" gestagen according to a standard procedure (2, 3).

The acetone- and ether-insoluble precipitate was re-suspended in a small volume of water, acidified to approximately pH 1, and hydrolyzed for six hours in a boiling water bath to release the "bound" gestagen. The latter was then extracted with ether, taken up in oil, and separately bio-assayed.

The procedure of Hooker and Forbes has been shown to detect not only progesterone but also its biologically active metabolites, Δ^4-3-ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol, and possibly other, unidentified gestagens as well (4). Gestagen levels may be expressed in Progesterone Equivalents (P.E.). One P.E. is defined as equal to the *activity* of 1 μg. of progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors.

RESULTS

"Free" Gestagen in Plasma. Gestagen was not detected in the plasma of the five mice injected with oil only.

Mice injected with 75 μg. of progesterone were bled at intervals of up to 20 minutes thereafter. Gestagen levels reached 3 P.E./ml. plasma in two mice and were less than 2 P.E./ml. in all others. Plasma concentrations of gestagen declined rapidly after the first five minutes.

Levels of gestagen in the plasma of mice receiving 2,250 μg. or more of progesterone usually fluctuated widely during the half hour in which they were studied. Levels reached a peak (as high as 8.3 P.E./ml. in an exceptional case) in ten minutes and then declined rapidly.

Plasma levels were followed for eight hours in mice receiving 1,500 μg. of progesterone. In the first hour, samples assayed as high as 9.1 P.E./ml. Thereafter, levels also fluctuated widely and erratically, ranging from 6.1 P.E./ml. to less than detectable amounts.

There was no consistent agreement between size of dose and plasma level of gestagen.

"Bound" Gestagen in Plasma. Forty specimens were assayed for "bound" gestagen. Again, plasma levels were not consistent with dose. The highest concentration observed was 4.4 P.E./ml. Usually the levels were much lower, and frequently no "bound" gestagen could be detected.

Gestagen Levels in Recovered Oil. No gestagen was detected in oil recovered from mice injected with oil only.

Results for the other mice are presented in Figures 1 and 2. A striking feature is the remarkable and consistent speed with which the hormone disappeared from the oil. Figure 1 shows that all values for the 75 μg. group and some for the 1,500 μg. group lie close to one curve, while all other values during the first hour lie close to a second curve. Statistical analysis of the values on the second curve showed that the amount of gestagen was reduced by an almost constant percentage for each unit of time. The rate at which gestagen left the oil in mice receiving the lowest doses of hormone, while relatively faster, also appears to be quite consistent. Whether the

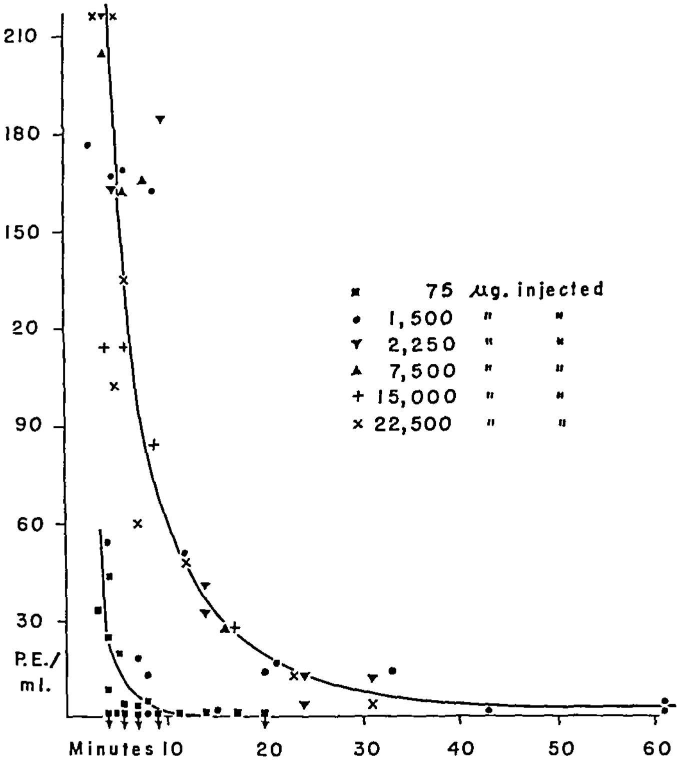

FIG. 1. Graph of assay results for oil recovered during first hour. Each symbol represents the sample from one mouse.

initial dose was 1,000 µg. or 15,000 µg./ml. oil, the concentration (assuming that no progesterone was transformed while in the oil to another compound of different activity) had fallen in ten minutes to approximately 60 µg./ml. oil and in 30 minutes to about 12 µg./ml.

Oil was recovered from mice receiving 1,500 µg. of progesterone not only during the first hour (Fig. 1) but also during the ensuing seven hours (Fig. 2). Thirty mice were killed after the first hour. Oil recovered from 20 of them assayed at low levels lying close to the already established curve. Hormone concentrations in samples from the other 10 mice were considerably higher, levels ranging from 9.5 to 27 P.E./ml. oil; most of the high values were in oil recovered after six hours. However, if the mean value for the second through sixth hour (3.7 P.E.; standard deviation of 2.8 P.E.) and that for the seventh and eighth hours (10.1 P.E.; standard deviation of 12.5 P.E.) are compared, the differences are not statistically significant.

DISCUSSION

The plasma levels of gestagen demonstrate that progesterone or its metabolites reached the blood stream within a few minutes after the former substance was injected intraperitoneally. Nevertheless, mere escape of

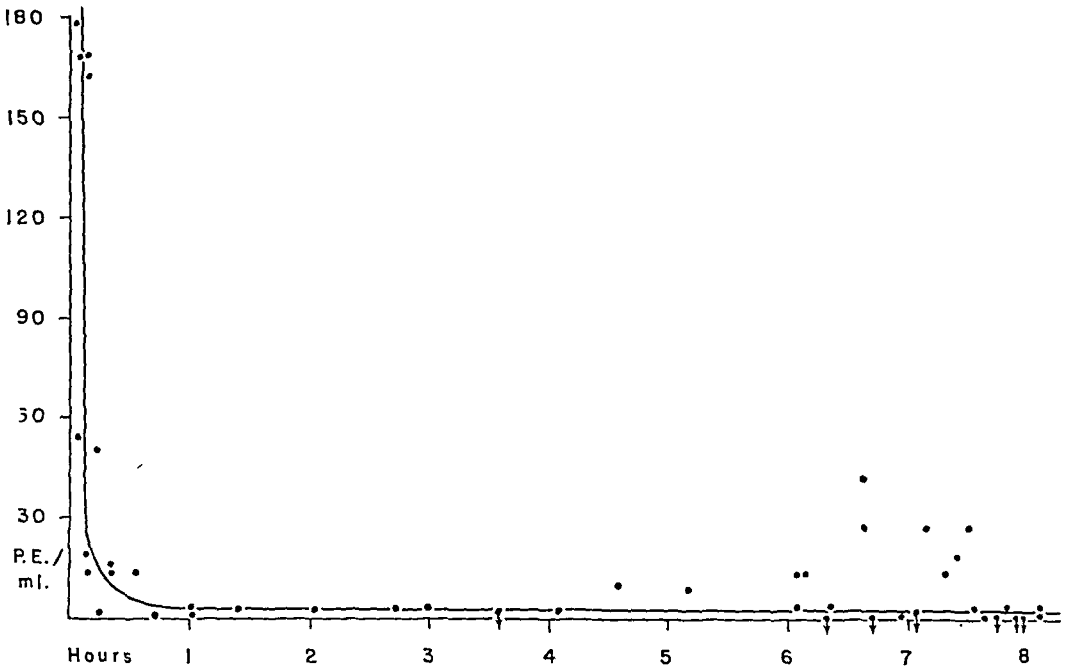

FIG. 2. Graph of assay results for oil recovered during eight hours in mice injected with 1,500 μg. of progesterone. Each symbol represents the sample from one mouse.

the progesterone into the plasma cannot in itself account for the hormone's swift disappearance from the oil. The total plasma volume of the young adult mouse is only perhaps 1 ml., and can contain at most only a few micrograms of gestagen.

Hepatic inactivation of progesterone occurs in the mouse (5), and undoubtedly accounted for the disappearance of some of the injected hormone. What happened to the remainder of the progesterone is not clear. The total area of the parietal and visceral peritoneum of the mouse is appreciable. It may be that much of the hormone adhered to or was absorbed by the peritoneal cells. It is possible that a part of the progesterone was taken up in the fat; concentrations of up to 2.3 and 4.4. μg. per gram of fatty tissue have been found in rabbits and women, respectively (6, 7). The fact that in some mice the concentration of the oil solution apparently rose during the seventh and eighth hours suggests partial return of the hormone to the oil. It is also possible that the administered progesterone, instead of leaving the oil, was transformed in the oil into a compound not detected by the bio-assay, but such transformation in this site appears unlikely.

The remarkable speed and seemingly steady rate at which the hormone escaped from the oil are interesting. Plotz and Davis (8) found that C^{14}-4-progesterone apparently moves rather quickly out of its oily solution after injection of the latter into human beings. In mice it appears that intraperitoneally injected concentrations of 50 to 1,000 μg./ml. oil are disposed of at one rate, and concentrations of 1,000 to 15,000 μg./ml. at another.

Data for the first curve were too scanty for statistical treatment. The second curve reveals a fairly uniform per cent loss per minute, implying that a mechanism relatively unaffected by biological variation may have been responsible for movement of the hormone out of the oil.

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A COMPARISON OF THE INHIBITORY EFFECTS OF 2-METHYL-1,2-BIS(3-PYRIDYL)-1-PROPANONE AND AMPHENONE B ON ADRENAL CORTICAL SECRETION IN THE DOG[1]

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ABSTRACT

In short term experiments in the dog 2-methyl-1,2-bis(3-pyridyl)-1-propanone (SU 4885) in high dosage totally suppressed the secretion of steroids by the adrenal gland. In low dosage there was a shift from cortisol and corticosterone to 11-desoxycortisol (Reichstein's Compound S) and desoxycorticosterone suggesting a selective inhibition of hydroxylation at C_{11}. In contrast amphenone suppressed total steroid secretion (*i.e.*, of cortisol and corticosterone) with no evidence for a selective action on 11-hydroxylation.

THE demonstration by Nelson and Woodard (1) that the administration of the insecticide DDD (2,2-bis (parachlorophenyl), 1,1-dichloroethane (Fig. 1) caused adrenal cortical atrophy in the dog, provided an indication that, at least in this species, the adrenal gland may be inhibited by a synthetic non-steroidal substance, although recent work by Cueto *et al.* (2) suggests that the ortho-para analogue was the active principle of impure DDD used in earlier experiments (1). Other related compounds such as Perthane (Fig. 1) have been studied by Taliaferro and Leane (3) and Larson *et al.* (4) who found that relatively minor changes in the chemical structure altered the action on the adrenal cortex.

The adrenal inhibitor which has been most extensively investigated is amphenone B (3,3-bis (p-aminophenyl)-2-butanone). Studies by Hertz (5) and other workers have shown that this substance inhibits adrenal steroid secretion both in the dog and in man, although the histological appearance of the adrenal cortex after the administration of the drug was not atrophy but hyperplasia. Recently, the compound 2-methyl-1, 2 bis (3-pyridyl)-1-propanone (SU 4885 or DPP) has been synthesized by Allen

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[2] Recipient of a British-American Exchange Fellowship from the American Cancer Society.

[3] Research Fellow in Medicine of the American College of Physicians, 1957–1958.

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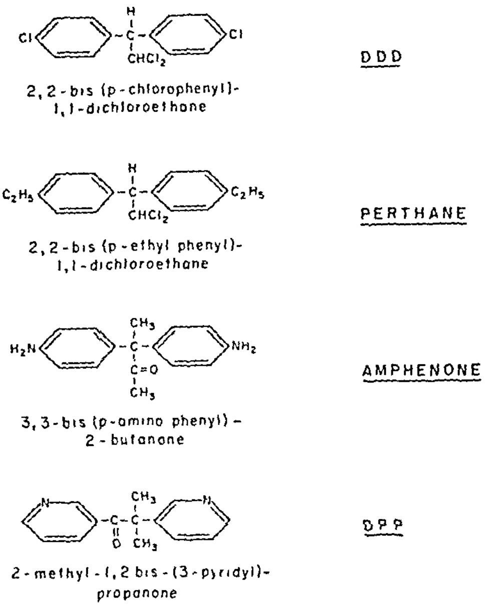
2,2-bis (p-chlorophenyl)-1,1-dichloroethane
DDD
2,2-bis (p-ethyl phenyl)-1,1-dichloroethane
PERTHANE
3,3-bis (p-amino phenyl)-2-butanone
AMPHENONE
2-methyl-1,2 bis-(3-pyridyl)-propanone
DPP

FIG. 1. Pharmacologic inhibitors of adrenal function (DPP=SU 4885).

and Bencze (Fig. 1) and preliminary results obtained by Chart *et al.* (6) indicated that this drug was effective as an inhibitor of adrenal steroid secretion in experimental animals. The ability of this substance to selectively inhibit oxygenation at the 11-position of the steroid molecule has been described (7, 8). The present report is concerned with an investigation of the effects of SU 4885 on adrenal cortical function in the dog and a comparison of these effects with those produced by amphenone.

METHODS

Mongrel dogs weighing 20–25 kg. were used. The right lumbo-adrenal vein was cannulated under sodium pentobarbital anesthesia according to the method of Nelson and Hume (9) and the adrenal venous blood was collected at timed intervals. An infusion of 25 units of hog ACTH in 500 ml. of normal saline was administered through the femoral vein, to ensure constant maximal adrenal cortical stimulation. Total 17-hydroxycorticoids in the blood were estimated by the method of Nelson and Samuels (10).

Characterization of individual steroids was carried out by extracting the plasma twice with equal volumes of ethyl acetate, washing first with 1N sodium hydroxide, then with water, drying with anhydrous sodium sulfate and evaporating to dryness. As much lipid material as possible was removed by partitioning the extract between hexane and 70% aqueous methanol. The methanol layer was removed, evaporated to dryness, redissolved in a methanol-chloroform 1:1 solution and applied to a paper chromatogram. The chromatograms were run in the "B3" and "B5" systems of Bush (11), and the toluene-propylene-glycol system of Zaffaroni (12). Steroids were identified by means of the running rates of the free and acetylated substances compared with known standards, ultraviolet light absorption, the soda fluorescence test (11), reactions with blue tetrazolium and phenylhydrazine reagents, and absorption spectra in concentrated sulphuric acid. Quantitation was carried out by eluting the steroids from the paper with methanol, and using the phenylhydrazine reaction of Porter and Silber (13) for the hydroxycorticoids and the blue tetrazolium method of Mader and Buck (14) for the other steroids.

RESULTS

When a single injection of 75 mg./kg. of SU 4885 bitartrate was given intravenously the total 17-hydroxycorticoids in the adrenal-venous blood fell to approximately 10% of the initial value within 10 minutes, rising to 75% within 3 hours as is shown in Figure 2. The effect of a small dose of the drug, 5 mg./kg. given as an infusion over 30 minutes is seen in Figure 3. In this case no decrease in 17-hydroxycorticoids occurred and in fact a suggestive rise was observed. When the infusion was increased to 75 mg./kg. a reduction in steroid output was again obtained.

The effect of a single intravenous injection of amphenone hydrochloride, in a molar dosage equivalent to 75 mg./kg. of SU 4885 bitartrate, is illustrated in Figure 4. The observed decrease in 17-hydroxycorticoids and the subsequent return toward the initial values was quantitatively similar to that obtained with SU 4885. It was noted, however, that during the course of the experiments, the anesthetic effect of SU 4885 was less than that of amphenone, since more pentobarbital was necessary to maintain the required state of anesthesia. Differences in the action of the two drugs became most apparent when the individual steroids were studied. A single injection of 75 mg./kg. of SU 4885 was given and the average of results from three experiments is seen in Figure 5. Cortisol fell from 7.0 μg. per minute to immeasurable levels within 10 minutes but even after 7 hours, the value was only 0.9 μg. per minute, returning to the initial level by 20

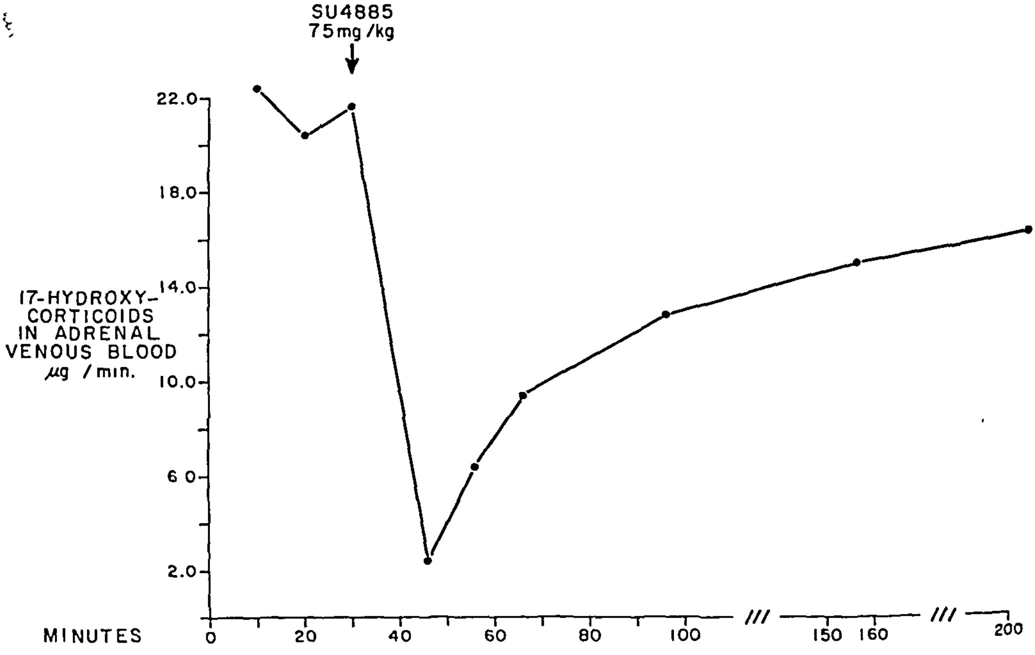

FIG. 2. The effect of a single intravenous injection of SU 4885 on total 17-hydroxycorticoid secretion in the adrenal vein of the dog.

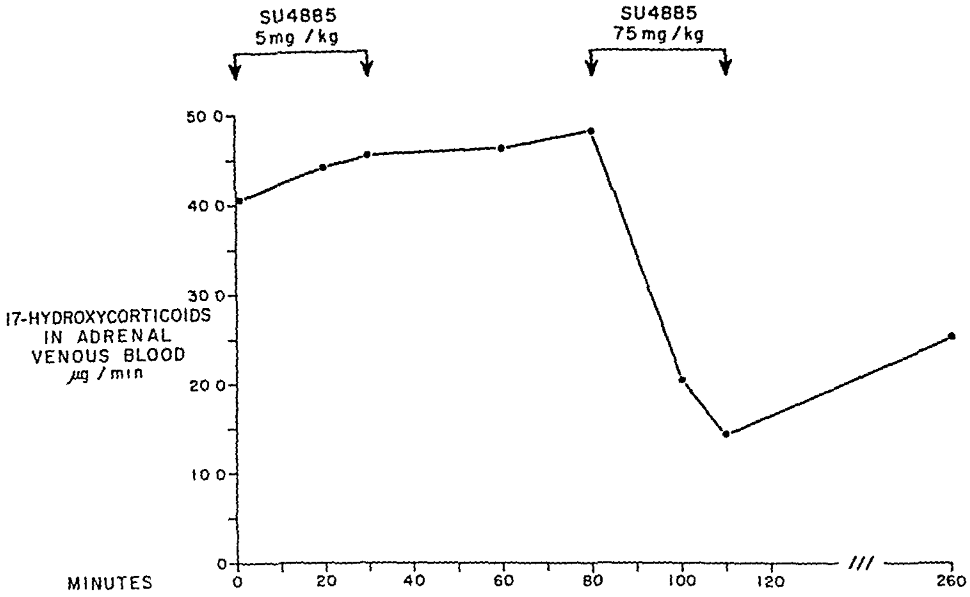

FIG. 3. The effect on total 17-hydroxycorticoid secretion in the dog of 30 minute infusions of SU 4885 in doses of 5 mg./kg. and 75 mg./kg.

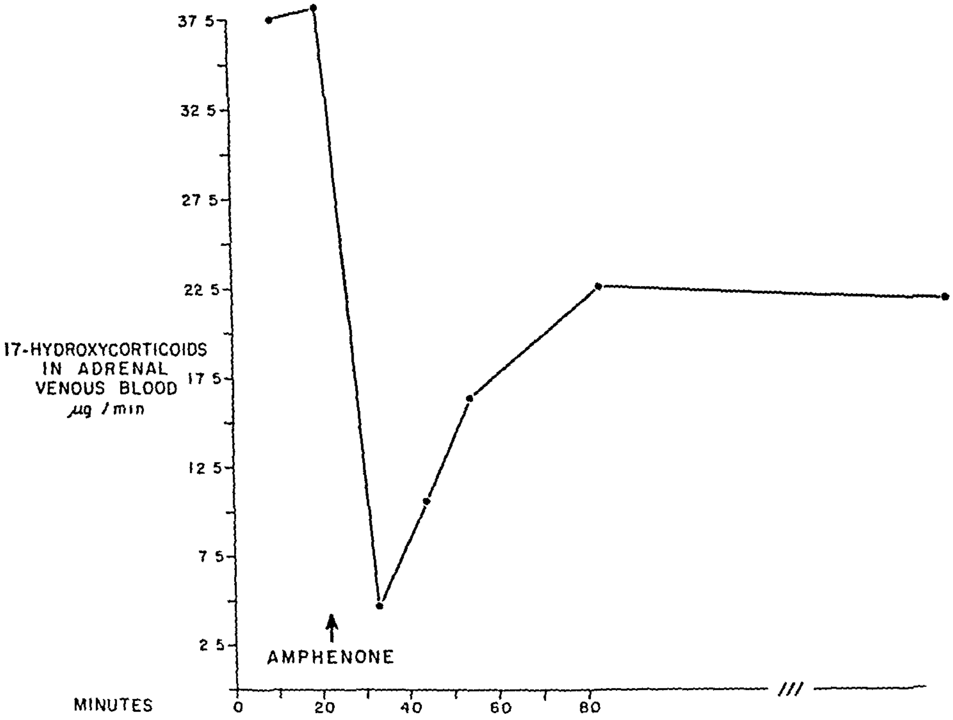

FIG. 4. The effect of a single injection of amphenone on total 17-hydroxycorticoids secreted into the adrenal vein of the dog.

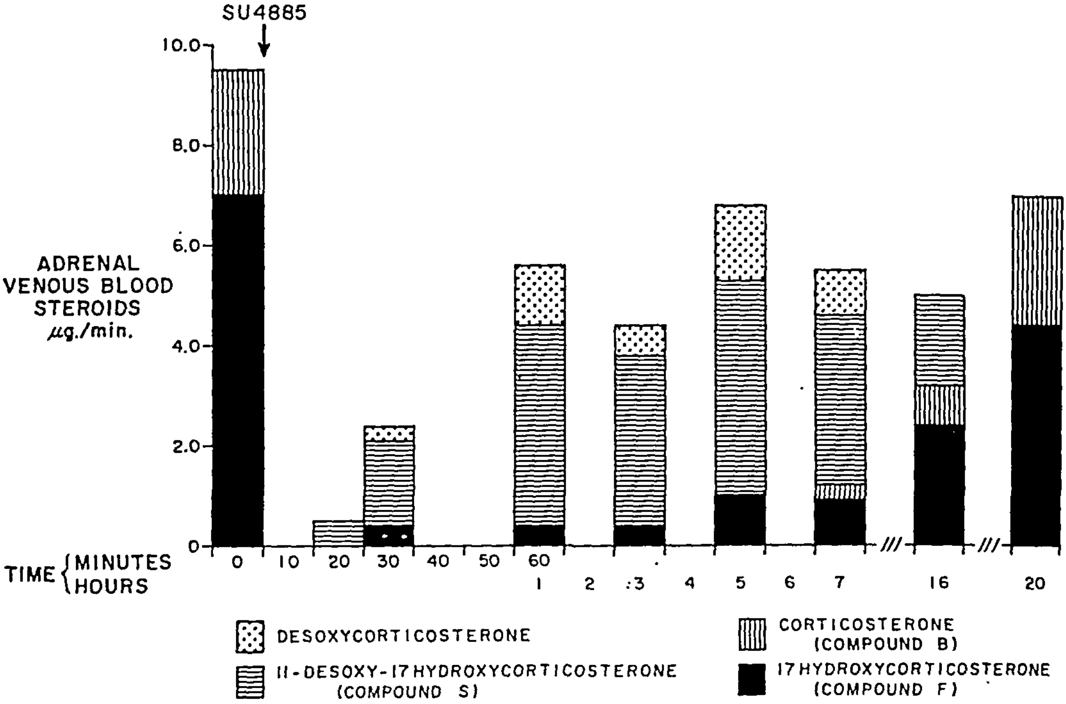

FIG. 5. Mean values for secretion of 17-hydroxycorticosterone (solid bars), corticosterone (vertical crosshatch), 11-desoxy-17-hydroxycorticosterone (horizontal crosshatch), and desoxycorticosterone (stippled) in three dogs given 75 mg./kg. SU 4885 intravenously in a single injection.

hours. Equally striking was the appearance of 17-hydroxy, 11-desoxy corticosterone (Reichstein's Compound S) which rose from previously undetectable levels to about 4 µg./min., 1–5 hours after the injection of the drug. It would appear that levels of Compound S are maximal by one hour and are maintained to at least 7 hours. Corticosterone, which was initially present in approximately half the quantity of cortisol disappeared and seemed to be replaced by 11-desoxycorticosterone.

In contrast with these results, Figure 6 shows the effect of amphenone in a dosage equivalent to 75 mg./kg. of SU 4885. A temporary reduction in cortisol and corticosterone was observed and during the following three hours the levels of both steroids gradually rose but without the appearance of 11-desoxy-compounds.

DISCUSSION

These findings indicate that SU 4885 has an inhibitory effect on adrenal cortical secretion in the dog. This action is not mediated by an interference with endogenous ACTH secretion since ACTH was administered during the course of each experiment. Quantitatively the effect on total 17-hydroxycorticoids seemed to be similar to that of amphenone, when given in an equivalent dosage, although the anesthetic effect of SU 4885 was

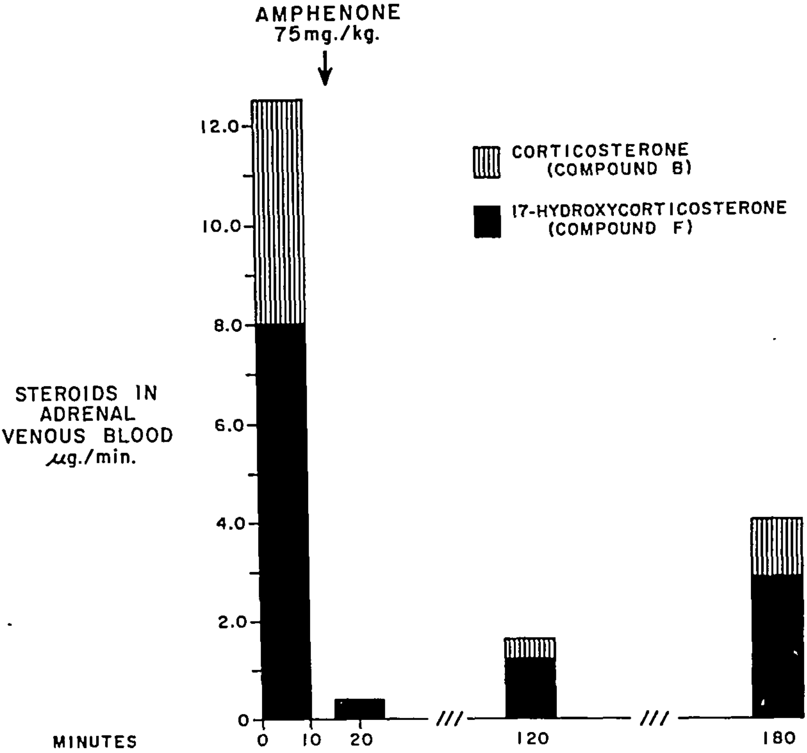

FIG. 6. The effect of a single injection of amphenone (75 mg./kg.) on secretion of corticosteroids in the dog.

less marked. The most important differences, however, were seen in the action of the two drugs on the individual steroids. In large doses SU 4885 caused a fall in total steroid secretion but this was followed by a prolonged inhibition of cortisol and corticosterone, lasting at least 7 hours, with the appearance of increasing amounts of Compound S and desoxycorticosterone. In smaller doses, no reduction in total 17-hydroxy steroids occurred but as was previously reported inhibition of cortisol occurred, with the concomitant secretion of large quantities of 11-desoxycortisol, indicating that the adrenal 11-hydroxylation enzyme system was especially sensitive to the effects of this drug (7, 8). The action of SU 4885 is in contrast with that of other known adrenal inhibitors. Studies with DDD (2) showed that reduction in 17-hydroxycorticoids in the dog following the administration of this drug was accompanied by actual destruction of adrenal cortical tissue. Amphenone (4) and SU 4885 (6) caused an increase in adrenal size. The experiments described above, demonstrate that amphenone produced a temporary suppression of cortisol and corticosterone but without evidence of any selective action on 11-hydroxylation. This is in agreement with the findings of Rosenfeld and Bascom (15), who showed, as a result of adrenal perfusion experiments, that amphenone caused a generalized inhibition of adrenal steroid secretion by interference with a number of different enzymes.

The selective action of SU 4885 is, therefore, of considerable interest, although the exact mode of its activity remains to be elucidated. Tomkins *et al.* (16) have presented evidence that the 11β-hydroxylase system is complex, at least three enzyme factors being involved, so that the drug could have one or several sites of action. Further study of the effects of this compound is indicated and may lead to increased knowledge of the mechanism of adrenal steroid biosynthesis.

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DEMONSTRATION OF THE CONCENTRATION OF ASTATINE-211 IN THE MAMMARY TISSUE OF THE RAT[1]

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ABSTRACT

Concentration of At^{211} in the mammary tissue of virgin and lactating female rats has been demonstrated both by gross radioactive assay and by autoradiography. Secretion of At^{211} in the milk was shown by its presence in the gastrointestinal tracts and carcasses of nurslings and by the presence of alpha-particle tracks in glandular lumens in autoradiographs. In virgin mammary tissue At^{211} was chiefly associated with the parenchyma, although small amounts could also be found in the stroma.

INTRODUCTION

WHEN 55-day old female Sprague-Dawley rats were given 0.5 μc./gm. body weight of At^{211}[2](1), 75% of the At-injected rats developed mammary tumors primarily of duct origin during the ensuing year. Only 17% of non-injected control rats developed mammary tumors during this same interval (2–3). In the elucidation of the cause of this augmented tumor incidence, it was of paramount importance to investigate the possible role of direct alpha-particle irradiation of the immature mammary tissue. There were indications from earlier work that At^{211} might concentrate in mammary tissue (4). The metabolism of At^{211} resembles that of I^{131} in many respects, and gross assays had shown that both halogens tended to concentrate in the skin and subcutaneous tissues (4). Further, Rugh (5) and Brown-Grant (6) have demonstrated the capacity of the mammary gland to concentrate I^{131} and to secrete it in milk.

Anatomically, the breast tissue of the virgin rat consists of a simple duct system terminating in tiny alveolar buds, embedded in, and surrounded by a copious amount of connective tissue and fat (7). Gross dissection of vir-

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[1] This work was performed under the auspices of the U. S. Atomic Energy Commission.

[2] At^{211} is an isotope of the heaviest halogen with a half-life of 7.5 hours. It decays by emission of alpha particles with a mean energy of 6.3 Mev and by electron capture (1). In the latter process 80 Kev x-rays are emitted allowing the detection of this isotope with photosensitive scintillation equipment.

ginal mammary parenchyma is virtually impossible; thus radioactive assay was inconclusive for assessing the concentration of At^{211} in mammary tissue. The At^{211} found in the mammary region might merely be associated with blood vessels, lymphatic tissue, or fat. Autoradiographs, however, provide a means by which the presence of At^{211} in the mammary parenchyma can be demonstrated visually. Because of the possibility of movement of At^{211} from its site of deposition in the living tissue during the processes of fixation and dehydration, it was also necessary to study the distribution of At^{211} in the lactating mammary gland and to determine whether At^{211}, like I^{131}, is secreted in the milk.

METHODS

At^{211} was prepared by the method of Parrott *et al.* (8). Three 43-day old virgin female Sprague-Dawley rats and two lactating Long-Evans rats each received 200 μc. of At^{211} intraperitoneally. After injection, the lactating rats were returned to their litters. Four hours later all the At-injected rats and the nurslings of the injected mothers were sacrificed with chloroform. This experiment was repeated with identical groups of virgin and lactating rats, except that the animals were sacrificed three hours after the At^{211} injection. Samples of mammary tissue were dissected from both inguinal regions of each of the injected rats, and were fixed in Bouin's fluid. One portion was dehydrated with dioxane, embedded in paraffin, sectioned at 6μ, and mounted on Eastman's 10μ NTA stripping film for autoradiographs. The remaining portions of the mammary tissue were weighed and assayed for At^{211} x-ray activity with a NaI-Tl crystal scintillation counter.

The gastrointestinal tracts and thyroids of 4 nurslings from the litters in Experiment 1 were dissected and assayed, and when present, bladder urine was also assayed. To exclude the possibility that any radioactivity present was due to external contamination, the remaining nurslings were skinned, and carcasses were assayed separately. In the second experiment all the nurslings were skinned and the gastrointestinal tracts dissected. These samples, and the residual carcasses were assayed separately.

RESULTS

The measurements of At^{211} radioactivity in mammary tissue of virgin and lactating rats are shown in Table 1. The 1-hour difference in post-injection interval did not affect the At^{211} concentration, percent of injected dose per gram of wet tissue (%/gm.), in the virginal tissue. The mean for all animals was 0.38%/gm. On the other hand, the At^{211} concentration in the lactating mammary tissue was appreciably higher at the earlier interval. This difference is reflected in the amount of At^{211} transferred to the nurslings at the 2 intervals (See Table 2). The total passed to the litters was twice as much 4 hours after injection as after 3 hours. At the 3-hour interval the gastrointestinal tracts contained 35% of the At^{211} found in the young. One hour later, while the total At^{211} in the nurslings had doubled, the gastrointestinal concentration was reduced to 25%, indicating rapid absorption of At^{211} from the infant gastrointestinal tract. The relatively low skin concentrations indicated that contamination from such sources

TABLE 1. DEPOSITION OF At^{211} IN TISSUE FROM THE MAMMARY LINE OF IMMATURE VIRGIN FEMALE RATS AND LACTATING RATS 3 TO 4 HOURS AFTER INTRAPERITONEAL INJECTION

Animal	Postinjection interval	Sample wt., Gm.	% injected At^{211}	At^{211} concentration %/gm.
Virgin rats				
a	3 hours	.25	.08	.31
b		.25	.14	.57
c		.68	.27	.40
d	4 hours	.35	.13	.36
e		.33	.10	.29
f		.49	.17	.34
Mean				.38
Lactating rats				
a	3 hours	1.69	1.47	.87
b		1.34	1.01	.75
c	4 hours	.95	.48	.50
d		.82	.32	.40

as the mother's urine was negligible, and that nearly all of the At^{211} found in the young had been transferred in the milk.

The distribution of At^{211} in the mammary glands was studied autoradiographically. In the glands from the virginal rats the tissue was preponderantly adipose. Scattered about within this stroma were small ducts and alveolar buds of epithelial structure. Small aggregates of lymphoid tissue were sometimes seen. The autoradiographs showed that the alpha-particle tracks most commonly arose from the epithelium, whether ductal

TABLE 2. MATERNAL TRANSFER OF At^{211} BY WAY OF THE MILK

Litter	Experiment 2 (3 hours)		Experiment 1 (4 hours)	
	No. of specimens	% of Mother's At^{211} dose	No. of specimens	% of mother's At^{211} dose
	a.		c.	
Whole nurslings	6	1.21	2	2.63
Skin only	6	.12	2	.32
GI Tract	6	.78	4	.90
Thyroid			4	<.01
Bladder urine			4	.02
Total passed to litter	(6)	7.3		15.8
	b.		d.	
Whole nurslings	6	.58	8	.84
Skin only	6	.07	8	.11
GI Tract	6	.33	4	.35
Thyroid			4	<.01
Bladder urine			4	<.01
Total passed to litter	(6)	3.5	(12)	10.1

(Fig. 1) or alveolar (Fig. 2). Tracks were very rare in the lumens of these structures. The adipose tissue also showed some At^{211}, but tracks were only about one-fifth as frequent. They arose from within the fat-containing space of the adipose cells. Tracks were also seen arising directly from the fibroblasts of the stroma. Blood vessels were scanty in this non-secreting gland, but tracks could sometimes be seen arising in the lumen of small vessels. The lymphatic tissue showed no tracks.

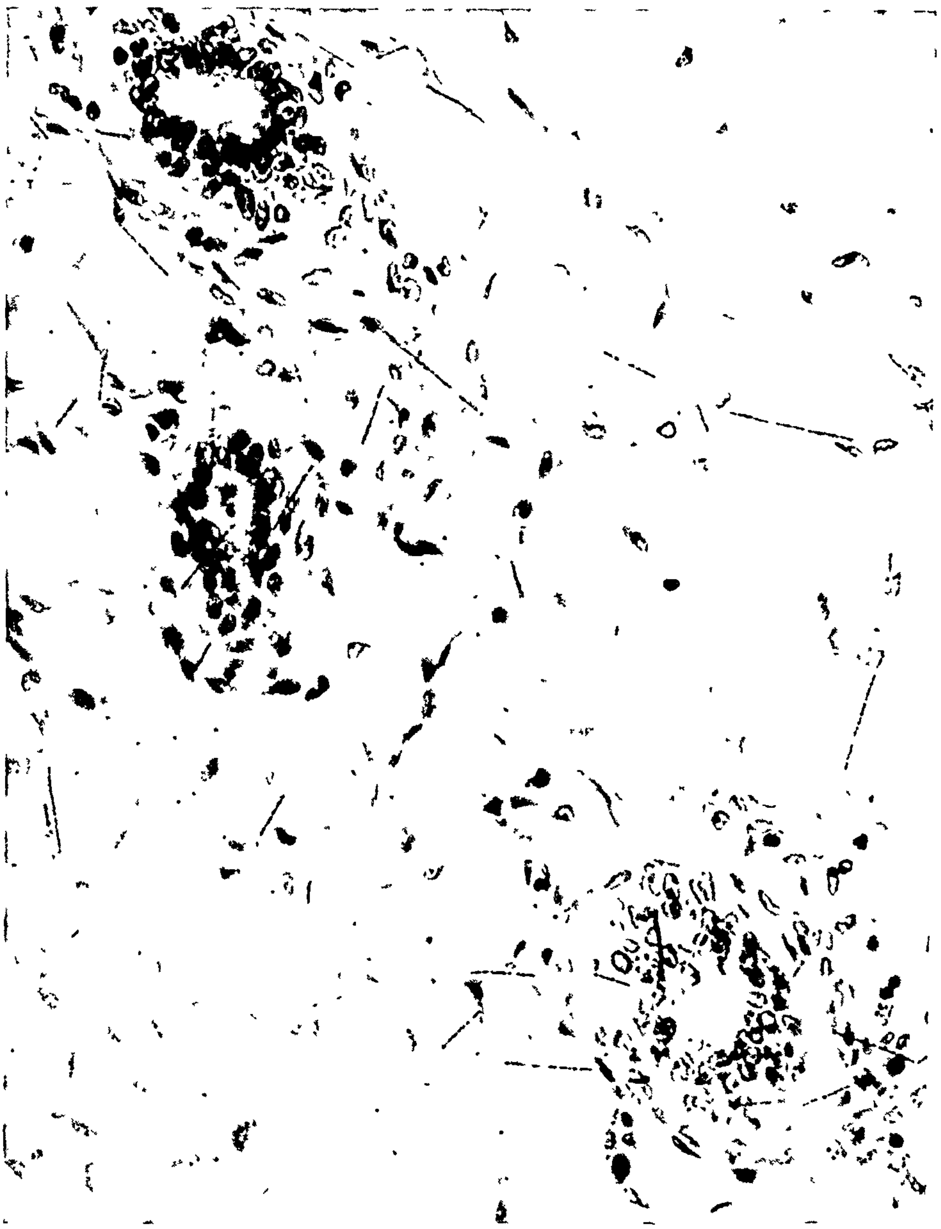

FIG. 1. Stripping film autoradiograph (NTA) of At^{211} localization in mammary tissue of 55-day old virginal rat. H and E counterstain; magnification 300×. Concentration in ducts and periductal tissue; sparse tracks in adipose connective tissue.

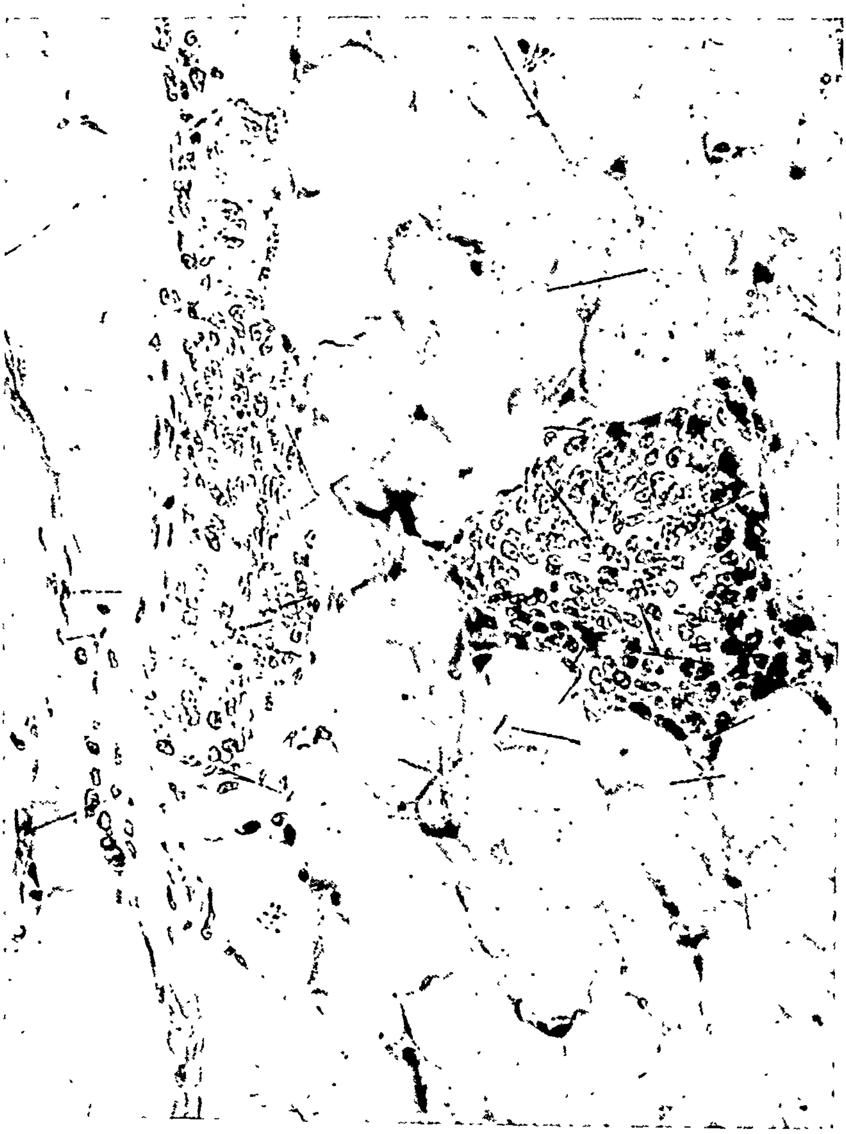

FIG. 2. Association of At^{211} with acinar tissue of mammary gland of 55-day old virginal rat. Technical data as in Figure 1.

In a typical autoradiograph from a lactating rat (Fig. 3) the tissue was predominantly glandular; the stroma contained an abundant vasculature and scanty adipose tissue. The acini of the glands were in varying stages of secretion, some being composed of heightened epithelium and a relatively small lumen, while in others the cells were lower, and the acini were dilated with secretion. In some acini tall cells were seen whose distal portion (i.e., toward the lumen) was distintegrating in the typical fashion of apocrine secretion. Lymphatic tissue could also be seen in these glands.

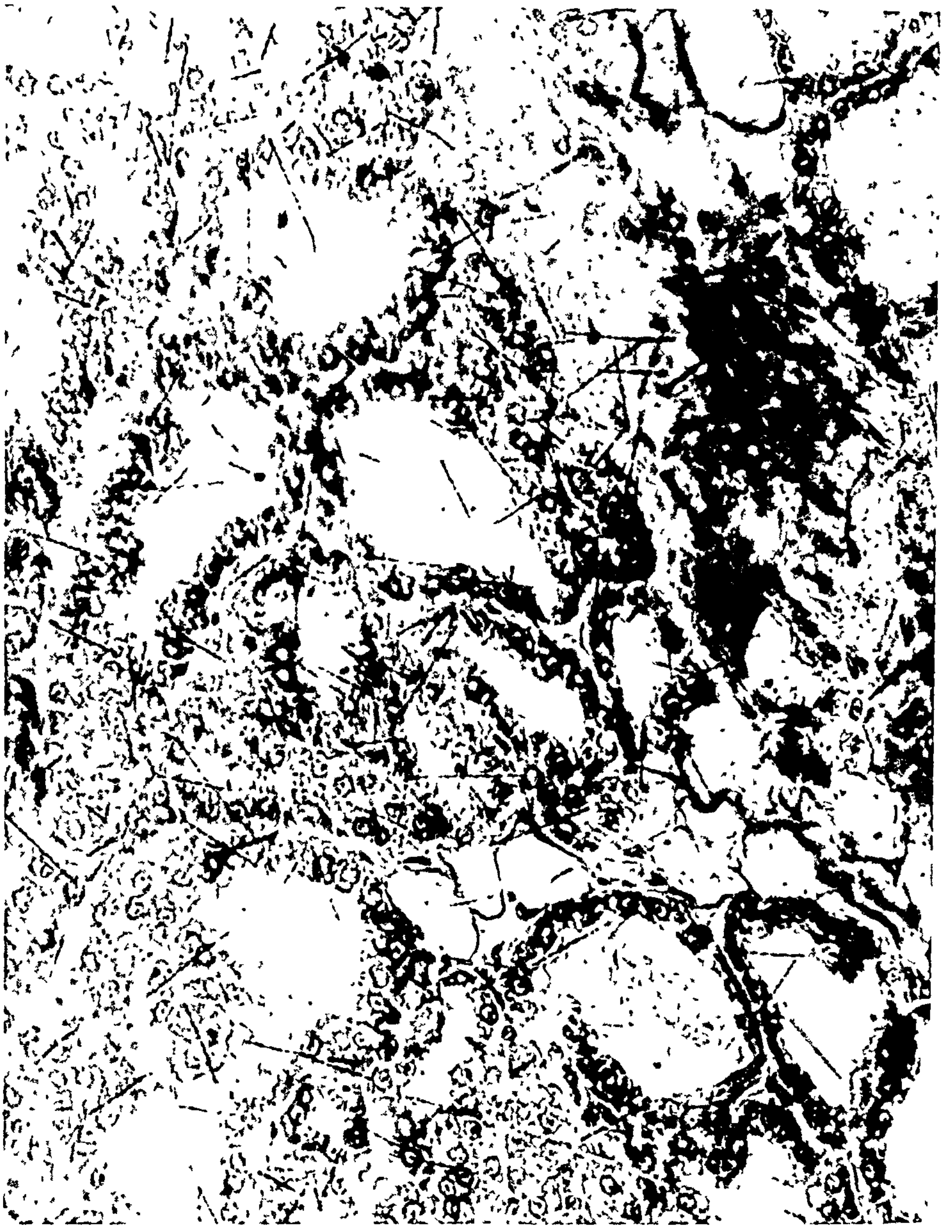

FIG. 3. At^{211} localization in mammary tissue of lactating rat. Note tracks in stroma, epithelium and lumens. Technical data as in Figure 1.

The autoradiographs showed tracks originating in the acinar epithelium. In the cells showing disintegration of the distal portion, tracks were often found originating in this part of the cell. Correspondingly, some tracks were seen in the lumen when it contained secretion. Tracks could also be seen arising from cells which were not yet actually releasing secretion. The blood vessels often showed a few tracks originating in the lumen and occasionally from the tissue of the walls. Tracks were rarely found in fat cells. The lack of tracks arising from lymphatic tissue of either group provided evidence that no appreciable translocation of the At^{211} had occurred during the technical manipulations.

DISCUSSION

The localization of At^{211} in lactating glandular tissue and its transfer to nursing young via the milk parallels demonstrations of radioiodine in milk. It is, of course, well established that casein may be iodinated *in vitro*, and it is possible that At^{211} is bound in comparable fashion. However, the possibility that it is associated with the fat of milk should not be neglected.

The epithelial localization in the non-lactating, virginal gland is of substantial interest in view of the frequency of occurrence of neoplasms of duct origin when such animals receive At^{211}. Of no less interest is the occasional uptake by fibroblasts in such animals, for fibromata and sarcomata are not infrequently seen, and tumors showing both epithelial and connective tissue hyperplasia are very common.

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THE EFFECT OF ADRENAL STEROIDS ON PERIPHERAL FAT MOBILIZATION IN THE RAT[1]

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ABSTRACT

The fasting adrenalectomized rat moved more fat out of the epididymal fat depot during a 12-hour fast than did the normal animal. Restoration of cortical hormone (hydrocortisone) at both high and low dose levels elicited a marked inhibition of fat movement out of this depot. The administration of hydrocortisone to intact animals also produced this inhibition. The procedure of sham adrenalectomy caused a significant diminution of fat mobilization from the epididymal depot for at least six days following the laparotomy. This response to trauma has now been observed in another laboratory following a burn stress. There are probably factors other than the release of cortical steroids which mediate this response but an increase in steroid titer appears to alter fat mobilization in this direction. The hypophysectomized animal behaves differently from the adrenalectomized animal in this regard. The amount of fat moved out of the epididymal depot by the hypophysectomized animal during a 12-hour fast was much smaller than that moved by either the intact or adrenalectomized animal. Administration of cortical hormones alone did not restore the rate of fat movement to normal. Work is in progress using combinations of target gland hormones and growth hormone in hypophysectomized animals.

ADRENALECTOMY inhibits the protein mobilization of stress (1) and the accumulation of fat by a damaged liver (2, 3). It has been suggested therefore, that adrenal insufficiency is accompanied by a simultaneous inhibition of fat mobilization out of peripheral fat depots. There are data which do not support this suggestion as regards depot fat movement during a fasting period (4, 5). Adrenalectomized rats appear to lose more rather than less fat from certain fat depots than do intact animals fasted for varying periods of time (6 to 48 hours) (5). We have investigated the effects of hydrocortisone on the total fat changes in the epididymal fat depot of the rat during fasting and feeding. Our data indicate that a plethora of cortical steroids inhibits fat movement out of this depot even during a fast in intact and adrenalectomized animals. Further, a deficiency of steroids augments fat movement.

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METHODS

Male Sprague-Dawley rats weighing 90–110 gm. were used throughout. Larger animals do not show comparable symmetry of the epididymal fat depots (5). In all experiments, each animal was used as its own control. The first epididymal fat body was removed immediately before the onset of the fasting period and the second at the end of the fast. Total lipids in each depot were determined by the method of Bloor (6). The difference between the amount of extractable lipid in the depots, before and after the fast, per 100 mg. of initial depot weight, was taken to indicate the degree of fat mobilization. The epididymal fat bodies were removed under ether anesthesia by orchiectomy through a lateral scrotal incision (5). All animals were maintained on Purina rat chow and water *ad libitum* with the exception of the adrenalectomized animals that were given 0.9% saline in place of tap water. Data are reported from only those adrenalectomized and hypophysectomized animals that did not show any adrenal or pituitary tissue on post mortem examination.

Intact, sham adrenalectomized, adrenalectomized (6 days postoperative) and hypophysectomized (11 and 217 days postoperative) rats were fasted for 12 hours and the total lipid content of the epididymal depots was determined.

Intact, sham adrenalectomized, and adrenalectomized rats were given either 0.3 mg. hydrocortisone or 1.0 mg. hydrocortisone subcutaneously for three successive days just prior to the start of a 12-hour fast. Hypophysectomized animals were given 0.3 mg. hydrocortisone one hour prior to the fasting period. Quantitative fat changes in the epididymal depot were determined.

RESULTS

Initial weight of the epididymal fat depot during ad libitum feeding: All animals were in the same weight range (90–110 gm.) when the first depot was removed. Despite this similarity in total body weight, there were significant differences in the amounts of fat deposited on the ad libitum feeding regime. In a total of 157 intact, untreated rats, the amount of extractable lipid from a single depot was 83.8 ± 1.5 mg. (S.E.). For 96 adrenalectomized animals the extractable lipid was 46.9 ± 1.7 mg. and for 59 sham adrenalectomized animals it was 69.2 ± 2.3 mg. Eleven days after hypophysectomy, the extractable lipid (8 animals) was 110.4 ± 7.0 mg. and in the series maintained for 217 days after hypophysectomy (8 animals) the lipid content was 187.9 ± 8.5 mg. It is apparent that adrenalectomized animals fed ad libitum do not deposit as much fat in this depot as do intact animals, while hypophysectomized animals deposit increasingly greater amounts than normal (Table 1).

The administration of hydrocortisone to intact, sham adrenalectomized and adrenalectomized animals for 3 days (during which time the animals were allowed free access to food and fluids) produced significant changes in the amount of fat deposited in the epididymal depots. At the two dose levels of hydrocortisone used in these experiments all the animals showed increased fat accumulation in the depot. The total body weight, however, did not change in the same direction. The animals did not show a significant weight gain and at the higher dose level of hydrocortisone (1.0 mg./day) many animals lost weight. In spite of this, the extractable lipids in a single depot in intact animals given 0.3 mg. hydrocortisone rose to 97.6 ±

TABLE 1. INITIAL WEIGHT OF EPIDIDYMAL FAT BODIES
(After *ad libidum* feeding)

Experimental procedure	No. of animals	Mg. of extractable fat	S.E.
Intact	157	83.8	1.5
Sham Adrenalectomy	59	69.2	2.3
Adrenalectomy	96	46.9	1.7
Hypophysectomy—11 days after operation	8	110.4	7.0
Hypophysectomy—217 days after operation	8	187.9	8.5
Intact—0.3 mg. hydrocortisone/day for 3 days	12	97.6	6.1
Intact—1.0 mg. hydrocortisone/day for 3 days	12	119.4	7.1
Sham Adrenalectomy—0.3 mg. hydrocortisone/day for 3 days	21	80.8	3.8
Sham Adrenalectomy—1.0 mg. hydrocortisone/day for 3 days	33	109.4	2.8
Adrenalectomy—0.3 mg. hydrocortisone/day for 3 days	19	75.8	3.5
Adrenalectomy—1.0 mg. hydrocortisone/day for 3 days	27	83.3	4.4

S.E. = Standard Error.

6.1 mg. With 1.0 mg. hydrocortisone, fat levels rose to 119.4 ±7.1 mg. as compared to the untreated animals' 83.8 ±1.5 mg. A similar increase was observed in the sham adrenalectomized animals. Thus, with 0.3 mg. hydrocortisone, the amount of extractable lipid was 80.8 ±3.8 mg. With 1.0 mg. hydrocortisone, the lipid content was 109.4 ±2.8 mg. as compared to the untreated sham adrenalectomized animals' value of 69.2 ±2.3 mg. The adrenalectomized animals responded to hydrocortisone in the same fashion. The untreated animals had 46.9 ±1.7 mg. of extractable fat in the depot. After treatment with 0.3 mg. hydrocortisone the amount of fat was found to be 75.8 ±3.5 mg. and after a regime of 1.0 mg. of hydrocortisone, was 83.3 ±4.4 mg. per depot (Table 1).

Changes in lipid content resulting from a 12 hour fast: It was found that intact animals fasted for 12 hours mobilized 13.0 ±2.4 mg. fat/100 mg. initial depot fat. Adrenalectomized animals mobilized significantly more fat during this period: 26.3 ±2.2 mg. fat/100 mg. initial depot fat. Hypophysectomized animals showed no significant mobilization of fat during this brief fasting period (Table 2). It is of interest that in these experiments the sham adrenalectomized animals also showed little or no quantitative fat changes in response to the withdrawal of food. A possible explanation for this will be discussed.

It can be seen from Table 2 that the administration of hydrocortisone to both intact and adrenalectomized animals effectively prevented the rapid mobilization of fat out of the depots that was observed in the untreated animals. It is difficult to evaluate the data obtained from the sham adrenalectomized animals because the procedure of sham adrenalectomy alone appears to inhibit fat mobilization within this time interval. The administration of hydrocortisone to hypophysectomized animals resulted

TABLE 2. MOBILIZATION OF EPIDIDYMAL FAT DURING A FASTING PERIOD

Experimental Procedure (12-Hour Fast)	No. of rats	Initial depot weight, mg.	Depot weight after, mg.	Mobilization mg.	S.E.	t	P	Mobilization, mg./100 mg. initial depot wt.	S.E.	t	P
Intact—saline	17	90.4	79.0	+11.4	2.4	8.1	<0.001	+13.0	2.4	9.5	<0.001
Intact—0.3 *mg. hydrocortisone*	12	97.6	115.1	−17.5	2.5			−18.2	2.5		
Intact—saline	17	90.4	79.0	+11.4	2.4	4.4	<0.01	+13.0	2.4	5.3	<0.01
Intact—1.0 mg. hydrocortisone	12	119.4	124.7	− 5.3	3.1			− 6.1	3.1		
Sham adrenalectomy—saline	16	70.8	69.9	+ 0.9	1.3	1.0	Not sig.	+ 2.5	2.8	1.3	Not sig.
Sham adrenalectomy—0.3 mg. hydrocortisone	11	80.8	76.8	+ 4.0	0.9			− 2.3	2.1		
Sham adrenalectomy—saline	16	70.8	69.9	+ 0.9	1.3	0.5	Not sig.	+ 2.5	2.8	1.4	Not sig.
Sham adrenalectomy—1.0 mg. hydrocortisone	20	109.4	106.5	− 2.9	0.8			− 3.3	3.0		
Adrenalectomy—saline	30	39.5	29.3	−10.2	1.4	5.0	<0.001	+26.3	2.2	7.0	<0.001
Adrenalectomy—0.3 mg. hydrocortisone	9	76.5	79.6	− 3.1	3.6			− 4.9	4.6		
Adrenalectomy—saline	30	39.5	29.3	+10.2	1.4	6.5	<0.001	+26.3	2.2	7.4	<0.001
Adrenalectomy—1.0 mg. hydrocortisone	15	75.0	76.0	− 1.0	1.8			− 2.7	3.0		
Hypophysectomy—11 days	8	110.4	105.8	+ 4.6	3.0			+ 3.5	6.3		
Hypophysectomy—217 days	8	187.9	191.8	− 3.9	5.6			− 2.0	5.6		

+ = Mobilization.
− = Deposition.
S.E. = Standard Error.

in ambiguous data and these experiments are being repeated with chronic rather than acute pretreatment with hydrocortisone.

DISCUSSION

Despite the accumulation of data concerning the metabolic effects of the cortical steroids, there remains uncertainty as to the mechanism of action of these hormones. For example, the regulation of fat metabolism during a fasting period has been attributed, in part, to the mobilizing action of the cortical steroids on the fat depots. This point of view is supported by data on protein mobilization (1) which suggests that the adrenalectomized animal cannot move protein from peripheral tissues to the liver when a caloric demand is imposed.

The fasting adrenalectomized animal is thus a more precarious animal in terms of available caloric substrate for metabolic needs. The hypoglycemia observed in such animals reflects this deficit. It has been suggested that this deficit is aggravated further by a concommitant defect in the availability of fat moieties from peripheral fat depots (7). The evidence for this is based largely on data obtained by measuring the degree of fat accumulation in the liver. It is clear that the adrenalectomized animal does not develop a fatty liver under circumstances which produce this effect in intact animals (2, 3). The level of liver lipid, however, is a resultant of many factors and does not always reflect the degree of fat mobilization from the depots. Thus, Gillman and Gilbert have found that in the baboon, the depletion of fat depots after adrenalectomy was not accompanied by the accumulation of fat in the liver (8). Earlier work from our laboratory also indicates that adrenal insufficiency produces a relative deficiency in protein mobilization while at the same time it increases the rate of fat mobilization (5). The level of liver fat does not mirror the peripheral fat depot behavior in this case.

In the experiments reported here, it was found that the adrenalectomized animal differs markedly from the hypophysectomized animal with regard to peripheral depot fat responses to feeding. Both types of preparations are known to resist fatty infiltration of the liver. In the case of the adrenalectomized animal, this phenomenon exists together with an augmented movement of fat out of the depot during fasting and a decreased fat accumulation in the depot during feeding, as compared to normal or sham adrenalectomized controls. On the other hand, the hypophysectomized animal consistently deposits more fat in the depot during a period of *ad libitum* feeding and moves fat out of the depot at a greatly reduced rate during a fast.

Stoerck and Porter (4), using large amounts of cortisone acetate (2 mg. daily) found that adrenalectomized animals showed a weight deficit but gained more adipose tissue. We used a relatively small dose of hydrocortisone (0.3 mg. daily) as well as a higher dose (1.0 mg. daily) and found that even at the lower dose range, this hormone prevented the net movement of fat out of the epididymal depot in normal and adrenalectomized animals in a 12-hour fasting period. The hypophysectomized animals without hormone treatment move so little fat out of this depot in a 12-hour fast that further inhibition would be seen only as an increase in fat deposition. There is some indication however, that under the circumstances of this complex hormonal deficiency (thyroxine, growth hormone, steroids) the administration of hydrocortisone at the low dose level may have augmented the movement of fat out of the depot. This is being investigated further. The work of Matthews *et al.* (9) suggested that there were marked metabolic differences in hypophysectomized animals kept for three months as compared to the acutely hypophysectomized preparation. We did not observe this in our procedures.

The behavior of the sham adrenalectomized animals with regard to depot fat changes was unanticipated. The adrenals were exposed, handled gently and the incision closed. Six days following this procedure, the animals were fasted and the usual depot fat measurements were made before and after the 12-hour fast. In more than 50 animals of this kind, it was found that there was a marked inhibition of the depot. The fasting, sham adrenalectomized animals behaved like the intact animals that had been pretreated for three days with hydrocortisone. The administration of hydrocortisone to the sham adrenalectomized preparation had no further effect on the rate of fat mobilization. We are investigating the time interval between operation and fasting during which this effect persists in the sham adrenalectomized animal. In addition, the length of the fasting period is being increased to 24 hours in order to increase the caloric deficit and thus increase the stimulus to fat mobilization.

In a recent paper by Milstein and Coalson (10) it was reported that the trauma of body surface scalding, evoked an inhibition of fat mobilization from a peripheral depot. This stress is known to produce an augmented protein catabolism. We observed a similar "sparing" of fat breakdown in the presence of rapid protein mobilization in both normal and adrenalectomized animals treated with phlorizin (5). The data on sham adrenalectomized animals is consistent with these findings. The stress of the surgical procedure also appears to elicit the conditions which tend to inhibit fat mobilization while augmenting protein breakdown. These effects persist for at least six days following the application of the stress in our experiments and in those described by Milstein and Coalson (10). A similar metabolic pattern may be imposed by the administration of hydrocortisone but there is little evidence to show that the titers of circulating corticoids remain significantly elevated for long periods of time following relatively mild stresses. The inhibition of fat mobilization under these circumstances probably involves factors other than steroid titers in blood. These factors are obscure at this time but warrant further investigation.

The use of the epididymal fat depot as an index of total body fat changes is susceptible of valid criticism. These data do indicate, however, that this depot is affected directly or indirectly by an elevation or diminution of cortical steroids. The direction of these effects is to slow the rate of fat movement out of the depot when steroid titers are raised. The influence of hydrocortisone on the increase in appetite may have accounted for the increased deposition of fat in the depot during the three-day period of pretreatment. As indicated, however, there was no evidence of a significant gain in total body weight as compared to the untreated controls. This again suggests that both large and relatively small amounts of this hormone increase fat accumulation while simultaneously decreasing protein synthesis (4).

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RE-EVALUATION OF THE EVIDENCE FOR A PITUITARY ERYTHROPOIETIC HORMONE[1]

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ABSTRACT

Evidence for the erythropoietic potency of adrenocorticotrophic preparations in the absence of the adrenal has been reexamined. Pituitary fractions of known erythropoietic activity did not correct the anemia in hypophysectomized rats when the adrenal was also absent.

THE anemia which follows hypophysectomy in the rat and the correction of this anemia or production of polycythemia, by the administration of pituitary extracts shows that anterior pituitary hormones participate in the adjustment between the erythrocyte content of the blood and the oxygen requirements of the organism. This paper reviews the evidence which led this laboratory to postulate the existence of a pituitary erythropoietic factor distinct from the well known pitutitary hormones. A reappraisal of the evidence in the light of recent experiments, has led to the abandonment of this postulation.

White and Dougherty in 1945 (1) described stimulation of erythropoiesis in mice by pituitary adrenocorticotrophic preparations, and a joint communication from the Division of Medical Physics and this laboratory in 1951 (2) reported that adrenocorticotrophic preparations prevented the development of anemia in hypophysectomized rats, and elevated the red cell volume in intact rats above normal values. Subsequently, groups of workers from these laboratories reported that corticotrophic preparations increased red cell volume in the absence of the adrenals (3, 4).

The finding that corticotrophic preparations increased red cell volume in the absence of the adrenal could not be reconciled with the hypothesis that adrenocorticotrophic hormone (ACTH) is the pituitary erythropoietic factor unless one assumes that ACTH acts in the absence of its target organ. This assumption appeared to be unwarranted. If ACTH could act in the absence of its target organ one would anticipate, for example, that adrenalectomized rats (maintained on salt) would develop polycythemia, inasmuch as the adrenal stimulating potency of the pituitary and of the blood plasma is greatly increased after adrenalectomy (5); actually adrenalectomized rats develop a mild, transient anemia (6).

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In cross transfusion studies in which hypophysectomized rats were parabiotically united with normal rats, the adrenal weights of the hypophysectomized partners were not significantly greater than those of hypophysectomized controls (7, 8). This suggested that only minute amounts of ACTH were transferred through the capillary interchange. However, when one of a normal parabiotic pair was subjected to hypoxia the erythropoietic effect was demonstrable in the partner (9). The interpretation drawn from these experiments was that ACTH and the erythropoietic factor were substances having different survival times in the circulation (4). Implicit in this interpretation was the assumption that the erythropoietic factor was of pituitary origin.

There were other evidences that erythropoiesis is regulated by extra-adrenal mechanisms, such as the erythropoietic response of adrenalectomized rats to reduced barometric pressure, or the erythropoietic response of normal rats to hypoxia without increase in adrenal size (4, 6, 10). Hypophysectomized rats, though limited in their response to hypoxia, nevertheless respond with increased red cell volume. Yet the adrenals remain very small and inactive (4, 11). Though a small amount of adrenal function undoubtedly persists in hypophysectomized rats, there is no evidence that hypoxia, or any other stimulus, increases adrenal function in the absence of the pituitary.

In an effort to determine whether the pituitary erythropoietic stimulus could be traced to any of the known pituitary hormones, the most highly purified preparations available were assayed for their erythropoietic action. It was known that growth hormone converts the bone marrow of the hypophysectomized rat from a pale, fatty tissue to a red, cellular one; however this hormone did not ameliorate the anemia, judged by hemoglobin concentration or red cell counts (12), or prevent the development of of post-hypophysectomy anemia as judged by red cell volume when expressed in terms of final body weight (13). Growth hormone was therefore not identified as an erythropoietic stimulant; although the total red cell volume was increased, it was not increased to a degree sufficient to ameliorate the anemia in the face of the increased body size. Purified thyrotrophic hormone partially corrected the anemic condition in hypophysectomized rats (14). Highly purified lactogenic hormone, almost free of contamination with ACTH (15), when injected in daily doses of 50 to 1000 μg. for 14 days failed to increase the red cell volume in anemic hypophysectomized rats (16). Beta-melanocyte stimulating hormone, containing 1000 u./mg. (17) had no effect on the red cell volume of the anemic hypophysectomized rat when injected in daily doses of 100 μg. in the 14 day assay (18). An increase in erythropoiesis following injection of pituitary gonadotrophins has not been demonstrated, though a stimulation might be anticipated from injection of interstitial cell stimulants on the basis of the reported effects of androgens on blood formation.

The anemia in the hypophysectomized animal has frequently been attributed to the loss of the trophic hormones, and the consequent atrophy of the target organs. Amelioration of the anemia has in fact been described after administration of testosterone and thyroxine given alone, and in combination (19, 20). Adrenal cortical steroids have also been implicated and have been reported to increase the number of circulating red blood cells in normal rats (21), though not in hypophysectomized rats except when given in combination with thyroxine (22, 23).

Thus purified growth and thyrotrophic hormones had some effect on erythropoiesis though they did not increase the red cell volume of hypophysectomized rats to normal values, whereas adrenocorticotrophic fractions were effective not only in the repair of the anemia of hypophysectomized rats but also produced polycythemia in normal rats. Since adrenocorticotrophic fractions appeared to be effective in the absence of the adrenal it was considered improbable that the erythropoietic component in such pituitary fractions was ACTH, and a separate pituitary erythropoietic factor was postulated. Therefore efforts were continued to separate chemically the erythropoietic principle from ACTH. The chemical procedures which were found to concentrate the erythropoietic factor were always those most useful in the preparation of ACTH and no clear separation could be accomplished. However, the erythropoietic and adrenocorticotrophic potencies did not always parallel one another. For example, pituitary fractions prepared by autolytic digestion, by digestion with added enzymes, or by chromatographic methods (4, 24), increased red cell volume 20–40% yet cause no increase, or a barely perceptible increase, in adrenal weight and no evidence of increased adrenal function as judged by thymic weight. In most instances such preparations caused some depletion in ascorbic acid, or slight changes in lipid content of the adrenal.

When highly purified ACTH[2] was assayed and found to be a potent erythropoietic stimulant, this finding though damaging to the concept of the separate existence of a pituitary erythropoietic factor, was not accepted as definitive evidence due to the difficulties inherent in determining purity of pituitary hormones. Such difficulties are well known and often originate from the confusing multiplicity of biological effects reported for hormones considered pure by current physico-chemical criteria. The work factor (26), melanocyte expanding activity (17), the fat mobilizing factor (25, 27), and the specific metabolic factor (28, 29, 30) have been or still are associated with highly purified preparations of ACTH.

Meanwhile through the studies of Borsook (31), Van Dyke (32), Gordon

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[2] Alpha-corticotropin was made available by C. H. Li and the assay here cited is referred to in his description of the biological properties of ACTH (25). Also tested were purified preparations of alpha-pool corticotropin from Lederle Laboratories, and corticotropin B from Merck and Co.

(33), Hodgson (34) and others, further knowledge has accumulated regarding erythropoietic factors in plasma and urine. Furthermore, Jacobson (35) and Crafts (36) and their collaborators have shown that such factors can be increased in the plasma of hypophysectomized animals by appropriate stimuli such as hypoxia or bleeding. The demonstration of a non-pituitary humoral factor invalidates the inference drawn from parabiotic studies regarding the separateness of the erythropoietic factor and ACTH. The erythropoietic response to hypoxia of the normal rat without adrenal hypertrophy, and of the adrenalectomized or hypophysectomized rat to this stimulus could be interpreted in terms of a similar extrapituitary humoral mechanism. The most cogent remaining evidence for the existence of a pituitary erythropoietic factor distinct from ACTH was the reported ability of pituitary fractions to stimulate erythropoiesis in the absence of the adrenal. The validity of this evidence has been re-examined.

In previous bio-assays of pituitary fractions in hypophysectomized-adrenalectomized rats difficulties had been encountered in maintaining the rats in good health, with resultant small numbers which survived the 14-day injection period. In efforts to determine a satisfactory regimen it had been found that substitution of 1% NaCl for drinking water was not adequate for maintenance after adrenalectomy combined with hypophysectomy. A larger proportion of the rats survived for the test period when given desoxycorticosterone acetate (DCA) in sesame oil, or desoxycorticosterone trimethylacetate in microcrystalline aqueous suspension. When DCA was combined with cortisol acetate the rats remained in even better condition. Due to the possible action of cortisol on body weight and red cell volume the latter steroid was not adopted for maintenance in all experiments.

Three series of experiments were performed in which corticotrophic preparations were tested for erythropoietic activity in hypophysectomized rats deprived of the adrenals. In the first two series, an oxycellulose eluate of sheep anterior pituitary was injected, in the third series alpha-corticotropin[3] was injected. In all series the preparations were assayed simultaneously in hypophysectomized rats.

The degree of anemia and its repair in hypophysectomized rats has been evaluated in this laboratory by determination of red cell volume using the Fe^{59} dilution method. This method though expensive and more time consuming than some other methods such as total red cell counts, reticulocyte counts, or hematocrit determinations, protects from errors introduced by possible changes in total blood volume. Due attention should be paid, however, to changes in body weight, or conditions which change the size or life span of the red cells (37). The test animal used in assay of erythropoietic potency of pituitary preparations was the female rat of the Long-Evans strain which was hypophysectomized at 26–28 days of age and used after a 45-day period. After this interval the post-hypophysectomy anemia has attained a reasonably stable value 40–50% below normal, the red cell volume (RCV) being on the average 1.40 to 1.50 ml./100 gm. BW. compared with the values in the normal rat of 2.30–2.40 ml./100 gm. BW. The pituitary fractions to be tested were suspended in beeswax and peanut oil and were injected subcutaneously daily for 14 days. Twenty-four hours after the last injection, red cell volumes were determined by the Fe^{59}-tagged red cell dilution method (38) which was briefly as follows: Labelled red cells were obtained from a rat previously injected with Fe^{59}. A small, known volume of blood containing Fe^{59}-tagged red cells of known specific activity was injected into the external jugular vein of the test animal under ether anesthesia. After 6 minutes to allow mixing of the injected sample in the recipient animal, a large sample of blood was drawn from the abdominal aorta into a heparinized syringe. The dilution of radioactivity was then determined in a blood sample of known volume. The red cell volume was calculated from these data and the hematocrit, and was then expressed in relation to body weight.

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[3] Alpha-corticotropin was kindly supplied by C. H. Li, of the Hormone Research Laboratory, Berkeley.

The oxycellulose eluate of sheep pituitary was prepared as follows. An aqueous extract of sheep pituitary was heated to 85° C. over an open flame with vigorous stirring

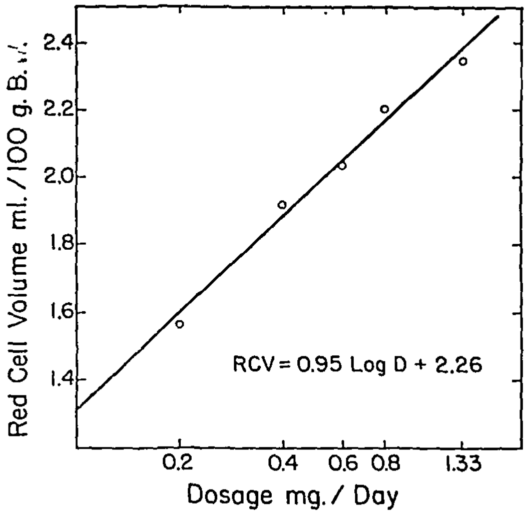

FIG. 1. Response of hypophysectomized female rats to graded doses of oxycellulose eluate. Each point represents the RCV of 15 to 30 rats.

The clear filtrate obtained from the coagulated suspension was brought to 0.1N by addition of glacial acetate acid (pH 3.8), was adsorbed on oxycellulose, eluted with 0.1N HCl, neutralized, dialyzed and lyophylized. The preparation was standardized carefully for erythropoietic activity in the anemic hypophysectomized rat before use in this series of experiments. The curve (Fig. 1) obtained from injection of increasing doses under standard assay conditions shows that the log dose-response relationship in hypophysectomized rats was linear.

The experimental animals were hypophysectomized at 28 days of age and injections were begun 51 days later. During this postoperative interval the rats were observed carefully, and any which appeared incompletely hypophysectomized (as judged by weight change, body tone, character of pellage or vaginal opening) were discarded. Rats which were also adrenalectomized were subjected to the second operation 50 days after hypophysectomy and injections were begun the next day. The adrenals when removed were carefully examined and no animals in which the cortex had been injured were included in experimental groups. At autopsy the completeness of hypophysectomy and adrenalectomy were determined by careful examination of the operation sites at 10-fold magnification. Any tissue not identifiable on gross examination was sectioned and stained appropriately. Cortical fragments less than 1 mm. in diameter were found in 6 of 62 adrenalectomized rats. Though the red cell volumes of these animals did not differ from those of completely adrenalectomized rats, these results were not included in the data presented. The adrenalectomized rats were not subjected to the usual terminal test for completeness of adrenalectomy, i.e., transfer to a high potassium-low sodium diet (6) as the blood withdrawal necessitated in determinations of blood volume invalidates this test.

Table 1 shows that the corticotrophic preparations caused no repair of the anemia in hypophysectomized rats deprived of their adrenals, although these same preparations were effective in restoring to normal the red cell volume and other hematological values in the hypophysectomized rats which were injected concurrently. The hematological values in hypophysectomized-adrenalectomized rats receiving ACTH and supported by steroids were somewhat higher than in untreated hypophysectomized rats, but these values were also higher in hypophysectomized-adrenalectomized rats and in hypophysectomized rats injected only with steroids. The increase is therefore not attributable to the action of ACTH.

These experiments do not confirm the earlier reports, and give no support to the concept that the erythropoietically active pituitary component is a separate hormone. The substance traced in chemical fractionations of pituitary tissue by its effect on red cell volume in hypophysectomized rats appears to have been the adrenocorticotrophic hormone. Although it is well established that the pituitary plays a role in regulation of erythropoiesis, there is no satisfactory evidence for a pituitary erythropoietic factor distinct from the known pituitary hormones. The adrenocorticotrophic hormone is clearly instrumental in the regulation of red cell volume, as is thyrotrophic hormone and possibly growth and gonadotrophic hormones. The relative importance and mechanism of action of the several factors remains to be determined.

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TABLE 1. ERYTHROPOIETIC ACTIVITY OF SHEEP OXYCELLULOSE ELUATE AND ALPHA-CORTICOTROPHIN IN HYPOPHYSECTOMIZED-ADRENALECTOMIZED ($\overline{A}\overline{H}$) AS COMPARED WITH HYPOPHYSECTOMIZED RATS

Series	Group	Age At			Maintenance‡	No. of rats	Body weight, gm.		Hematocrit %	Hemoglobin, gm./100 ml.	Total blood volume, ml.	Red cell volume, ml./100 gm. BW	Adrenals, mg.	Thymus, mg.
		$\overline{H}$ da.	$\overline{A}$ da.	Onset inj., da.			Initial	Final						
I*	$\overline{HA}$ Inj.	28	78	79	DCA 50 µg./da.	5	82	91	31.4±1.4	9.4±0.2	4.13±0.34	1.43±0.14	—	121
	$\overline{HA}$ Cont.	28	78	—	DCA 50 µg./da.	7	82	84	29.1±1.1	9.1±0.3	4.25±0.17	1.47±0.08	—	109
	$\overline{H}$ Inj.	28	—	79	—	5	78	91	42.6±1.4	12.4±0.5	4.93±0.25	2.31±0.07	21	<25
	$\overline{H}$ Inj.	28	—	79	DCA 50 µg./da.	5	77	80	42.3±5.2	10.7±1.2	4.61±0.32	2.44±0.39	29	<25
	$\overline{H}$ Cont.	28	—	—	—	5	79	88	24.1±2.1	7.7±0.7	4.04±0.14	1.11±0.11	8	113
II*	$\overline{HA}$ Inj.	28	78	79	DCA 50 µg./da.	2	75	73	33.9±0.2	11.0±0.0	3.65±0.59	1.70±0.05	—	87
	$\overline{HA}$ Inj.	28	78	79	Cortisol 50 µg./da. DCA 50 µg./da.	8	75	81	36.4±1.5	11.5±0.5	4.21±0.22	1.89±0.06	—	73
	$\overline{HA}$ Cont.	28	78	—	DCA 50 µg./da.	3	77	77	28.1±1.5	9.5±0.2	3.96±0.10	1.45±0.07	—	68
	$\overline{HA}$ Cont.	28	78	—	Cortisol 50 µg./da. DCA 50 µg./da.	9	73	77	34.3±1.1	10.6±0.4	3.90±0.15	1.74±0.07	—	63
	$\overline{H}$ Inj.	28	—	79	—	5	72	82	40.8±0.8	12.1±0.3	4.77±0.21	2.37±0.17	25	<25
	$\overline{H}$ Cont.	28	—	—	—	5	74	80	27.8±0.8	9.5±0.2	4.08±0.26	1.42±0.04	7	102
III†	$\overline{HA}$ Inj.	28	75	76	DCA (trimethyl) 1.25 mg. Cortisol 50 µg./da.	11	74	75	39.0±1.4	12.4±0.5	3.45±0.09	1.79±0.07	—	57
	$\overline{HA}$ Cont.	28	75	—	DCA (trimethyl) 1.25 mg. Cortisol 50 µg./da.	11	72	77	36.1±1.3	11.3±0.4	3.93±0.14	1.84±0.06	—	58
	$\overline{H}$ Inj.	28	—	76	—	5	70	78	40.8±2.3	11.5±0.3	4.58±0.21	2.40±0.16	31	<25
	$\overline{H}$ Cont.	28	—	—	—	5	71	81	30.2±1.5	9.4±0.3	3.88±0.17	1.45±0.06	8	108

* Injected groups received 1.3 mg. oxycellulose eluate suspended in 0.1 ml. of 4% beeswax in peanut oil subcutaneously, daily for 14 days.

† Injected groups received 25 µg. alpha-corticotrophin suspended in 0.1 ml. of 5% beeswax in peanut oil subcutaneously, daily for 14 days.

‡ Desoxycorticosterone acetate (Ciba) was given in a 0.05 ml. sesame oil subcutaneously, daily for 14 days.
Desoxycorticosterone trimethyl acetate (Ciba) was given in a single subcutaneous injection in 0.9% NaCl on the day of adrenalectomy.
Cortisol (Merck hydrocortone acetate) was given in 0.1 ml. of 0.9% NaCl subcutaneously daily for 14 days.

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CONVERSION OF C^{14}-TESTOSTERONE TO C^{14}-ESTROGENIC STEROIDS BY ENDOCRINE TISSUES[1]

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ABSTRACT

The ability of stallion testis, human term placenta and a human feminizing adrenal cortical carcinoma to convert C^{14}-testosterone to C^{14}-labeled estrogenic steroids has been demonstrated. The relationship between this conversion and the biosynthesis of estrogens by these tissues has been discussed.

STUDIES previously reported from these laboratories demonstrated the conversion of testosterone-3-C^{14} to C^{14}-estradiol by human ovarian slices (1). Similar results have been obtained by Wotiz, *et al.* who reported the formation of C^{14}-labeled estrone and estriol as well as estradiol from C^{14}-testosterone incubated with human ovarian tissue showing cortical stromal hyperplasia (2). In addition to these studies Heard, *et al.* have found that estrone-C^{14} is a urinary metabolite of testosterone-4-C^{14} in the pregnant mare (3). Meyer has shown the formation of estrone from 19-hydroxy-4-androstene-3,17-dione in endocrine tissue and has suggested that the 19-oxygenated compound is an intermediate between the C_{19} neutral steroids and C_{18} aromatic steroids (4).

There is no direct evidence that this pathway from testosterone to estrogens is the major one operating in the endocrine tissues producing estrogens. Some further indirect evidence bearing on this point can be

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furnished by studies of the ability of various tissues to carry out the conversion of testosterone to estrogenic steroids.

The results presented in this paper demonstate the ability of three additional types of endocrine tissues, all thought to produce estrogens, to carry out the conversion. These tissues are stallion testis, a human adrenal carcinoma, and a human placenta.

EXPERIMENTAL

Counter-Current Distributions

All 49- and 99-transfer counter-current distributions were carried out in a 100-tube glass Craig counter-current distribution apparatus (H. O. Post Scientific Apparatus Company). Unless otherwise indicated, equal volumes, 10 ml. each, of upper and lower layers were used for each transfer. The fractions were evaporated under reduced pressure with constant agitation, or from the frozen state.

Short distributions, four or eight transfers, were carried out in separatory funnels, lower layers being transferred.

Determination of Radioactivity

Samples for radioactivity analysis were plated directly in stainless steel counting dishes (1 inch in diameter, $\frac{5}{16}$ inch in depth) from organic solvents, usually 95% ethanol. All samples were essentially infinitely thin, less than 0.3 mg./cm.^{2} They were counted in a windowless flow counter. At least 2000 counts were accumulated and all counts reported are corrected for background.

Determination of Estrogens

The counter-current distributions were analyzed for estrogen by the method of Engel *et al.* (5), measuring fluorescence at 488 mμ after heating in H_2SO_4 (6). A Farrand photofluorometer was utilized for the measurement of fluorescence.

Tissue Incubations and Extractions

Stallion Testis. A single testis (65 gm.) removed from a four-year old Palomino[5] was immediately homogenized for two minutes in a Waring blendor with 120 ml. of 0.85% NaCl solution. The homogenized mixture was centrifuged at low speed and most of the supernatant fluid was decanted. The residue was suspended in 50 ml. of 0.06 M phosphate buffer (pH 7.4) containing 0.075 mM of sodium fumarate, 0.75 mM of nicotinamide, and 0.5 mM of $MgCl_2$. The homogenized tissue was divided equally among five 125 ml. Erlenmeyer flasks each containing 1 mg. of testosterone-3-C^{14} (490,000 cpm/mg.) dissolved in 0.1 ml. of propylene glycol. Incubation was carried out at 37° for 3 hours with shaking, using air as the gas phase.

The contents of the five flasks were combined and 5.0 mg. of estrone were added. The mixture was dried from the frozen state and the residue extracted in a Soxhlet extractor as described by Ryan and Engel (7).

Adrenal Carcinoma. This tumor, which weighed 2 Kg., was removed from a 66-year old male showing marked gynecomastia. His preoperative urinary 17-ketosteroid level was 510 mg./24 hr. (8). Paper chromatographic (9) examination of the extract disclosed a high proportion of dehydroepiandrosterone. Excretion of estrogens was also markedly elevated. The daily excretion of estrone was 187 μg.; estradiol, 47 μg.; and estriol, 170 μg.

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[5] We wish to thank Mr. Dwight Jacobs, Lynbrook Farm, Southborough, Massachusetts, for his cooperation in making this tissue available.

(5). Levels of 17-ketosteroids and estrogens fell to low levels post-operatively, but subsequently increased with growth of metastases.

A portion of the tumor was sliced in a Stadie slicer (0.5 mm. thickness) and 50 gm. of slices were added to each of two 750 ml. Erlenmeyer flasks containing 100 ml. of high potassium medium (10), 300 mg. glucose, 0.1 mM of potassium fumarate, and 0.23 mg. of testosterone-3-C^{14} (2.35×10^6 cpm/mg.) in 0.2 ml. of propylene glycol. The flasks were flushed with 95% O_2—5% CO_2 and incubated with shaking at 37° for 3 hours. Ten mg. each of estrone, estradiol, and estriol as well as several neutral steroids were added to the incubated mixture which was then homogenized in a Waring blendor and extracted with a mixture of methanol and chloroform according to a modification of the method of Folch (11).

Human Placenta. Tissue from a human term placenta was sliced in a Stadie slicer (0.5 mm. thickness), and 7.5 gm. of slices were incubated in 18 ml. of human serum enriched with 0.018 mM of potassium fumarate and 10 mg. of glucose and containing 0.23 mg. testosterone-3-C^{14} (2.35×10^6 cpm/mg.) previously dissolved in 0.2 ml. propylene glycol. The flask was flushed with 95% O_2—5% CO_2 and incubated 3 hours at 37° with shaking.

Two mg. each of estrone, estradiol and estriol were added to the incubation mixture as carriers along with 10 mg. each of several neutral steroids. The mixture was homogenized in a Waring blendor and extracted repeatedly with ethyl acetate, and ether:chloroform (4:1), and then with 10 volumes of acetone (12). Finally the residue was extracted three times with 20 volumes of chloroform:methanol (2:1). The combined extracts were evaporated to dryness. The residue was dissolved in chloroform and washed with 1/10 volume of water.

Preliminary Purification of Crude Extracts

Each of the extracts was subjected to an 8 transfer counter-current distribution between hexane and 90% methanol. In each case there was a peak in radioactivity in funnel 8 which contained the most polar material. In each experiment the contents of funnels 5–8 contained the bulk of the radioactivity and the carrier steroids present in the crude extract and were therefore combined. These polar fractions were next subjected to phenolic separations. In the case of the material from the incubations with stallion testis and the adrenal tumor, phenolic separations using toluene and IN NaOH were carried out (5), followed by 8-transfer distributions in the same solvent system (1). In both cases there were peaks in activity in the NaOH ends of the distributions. In the case of the fraction from the incubation with placental tissue, the phenolic material was separated by passing the fraction dissolved in methanol through an ion exchange resin (Nalcite SAR-8) in the bicarbonate cycle and washing the resin with additional methanol to remove neutral material.[6] The phenolic material was then eluted with 5N acetic acid in methanol. This procedure was repeated on the phenolic fraction from the first column.

The phenolic fraction from the incubation of stallion testis contained 2410 cpm; the one from the incubation of the adrenal carcinoma, 9730 cpm; and the one from the incubation of placenta, 316,000 cpm.

Separation and Purification of Estrogenic Steroids

Stallion Testis. Two mg. of estradiol were added to the phenolic material and the mixture subjected to a 99-transfer counter-current distribution in 70% methanol-30% H_2O/40% $CHCl_3$—60% CCl_4.

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[6] Engel, L. L. Unpublished data.

There was a small peak in radioactivity in the same tube as the peak in estrone ($K^7 = 0.21$); however, the level of radioactivity was too low to permit accurate measurement or further study. A peak in radioactivity also coincided with the peak in estradiol ($K = 1.04$); however, this region was contaminated by radioactivity slightly more polar than the estradiol. The contents of the tubes containing the estradiol (1.88 mg.) were pooled and subjected to a 49-transfer counter-current distribution in a solvent system consisting of 30% ethyl acetate—70% cyclohexane/50% ethanol—50% water.

Statistical analysis of the second counter-current distribution in the estradiol region for 1.5θ tubes (13) indicated radiochemical purity, the p value being greater than 0.2 and the coefficient of variation for specific activity, 0.11 (14). However, the counter-current distribution curve indicated that a more polar impurity overlapped the estradiol slightly. The specific activity was 400 ± 44^8 cpm/mg.

Adrenal Carcinoma.—The phenolic fraction was subjected to a 99-transfer counter-current distribution in 30% ethyl acetate—70% cyclohexane/50% ethanol—50% H_2O. Peaks in radioactivity coincided with the peaks in estrone and estradiol. The mean specific activity of the estrone ($K = 1.75$) in the region of the peak was 1260 ± 40 cpm/mg. The estradiol peak ($K = 0.78$) had a mean specific activity of 864 ± 21 cpm/mg. There was no peak in radioactivity associated with the peak in estriol ($K = 0.053$).

Placenta. The phenolic material was subjected to a 99-transfer distribution in 70% methanol—30% H_2O/40% $CHCl_3$—60% CCl_4. A peak in radioactivity conicided with the peak for estrone ($K = 0.22$). It contained 0.40 mg. of estrone and 82,300 cpm. The experimental points for radioactivity did not fit the theoretical distribution curve, indicating a lack of purity. The estradiol peak also corresponded to a peak in radioactivity ($K = 1.02$). This peak contained 0.42 mg. of estradiol and 79,100 cpm. As in the case of the estrone, the estradiol was not radiochemically pure. There was considerable radioactivity in a peak slightly more polar than the peak in estriol ($K = 4.82$). This radioactivity overlapped the estriol peak, thus obscuring any peak that might have coincided with the estriol. The contents of tubes 0–64, containing all the estrone and estradiol, were combined; contents of the remaining tubes, 65–99, containing the estriol and the polar radioactive compound, were also combined.

To the pool containing the estrone and estradiol from the preceding distribution was added an additional 10 mg. each of estrone and estradiol. The mixture was then subjected to a 170-transfer counter-current distribution in a solvent system consisting of 70% methanol—30% H_2O/20%

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[7] Partition coefficient, $K = $(concentration in upper phase)/(concentration in lower phase).

[8] Standard deviation.

$CHCl_3$—80% CCl_4. Peaks of radioactivity were associated both with the peak of estrone and that of estradiol; however, neither compound was yet radiochemically pure.

The estrone from the distribution of estrone and estradiol was subjected to a 99-transfer counter-current distribution using 40% ethyl acetate—60% cyclohexane/60% ethanol—40% water. A single peak of radioactivity and fluorescence (K = 1.02) was obtained. Statistical analysis of the data indicated radiochemical purity of this substance. The estrone had a specific activity of 5041 ± 625 cpm/mg.

The estradiol region from the 170 transfer counter-current distribution of the combined estrone and estradiol was pooled. This material was subjected to a 99-transfer distribution in 30% ethyl acetate—70% cyclohexane/50% ethanol—50% H_2O. A single peak of radioactivity and fluorescence was observed (K = 0.71). Statistical analysis indicated radiochemical purity. The estradiol had a mean specific activity of 4375 ± 644 cpm/mg.

The estriol from the first distribution was diluted with an additional 10 mg. of unlabeled estriol and subjected to a 99-transfer counter-current distribution between 50% ethyl acetate—50% cyclohexane and 33% ethanol—67% H_2O. The peak in estriol was associated with a much broader peak in radioactivity. The contents of the tubes around the peak of estriol were pooled. These contained 65% of the total amount of carrier estriol in the peak. This estriol was subjected to a third counter-current distribution. Ninety-nine transfers were applied in a solvent system consisting of 70% methanol—30% H_2O/60% $CHCl_3$—40% CCl_4. The volumes of the upper phases were 5 ml. for each tube, one-half the volume of the lower phase. The peak in estriol (K = 2.60) appeared to be associated with a peak in radioactivity. However, a second peak in radioactivity (K = 1.60) overlapped the peak in estriol, thus preventing an evaluation of its radiochemical purity.

The tubes containing 95% of the carrier estriol were combined and subjected to a 250 transfer counter-current distribution with recycling in 70% methanol—30% H_2O/60% $CHCl_3$—40% CCl_4, using a phase volume ratio of 0.5. This separated the less polar contaminant from the carrier estriol. However, statistical analysis of the estriol region indicated that the radioactivity has been separated from the carrier estriol and that the displacement of the two peaks was 1.4 tubes.

In order to substantiate this observation, the material in the region containing the carrier estriol was methylated by the procedure of Brown (15) and the product was subjected to a 99-transfer distribution, using 50% methanol—50% H_2O/carbon tetrachloride as solvent. The carrier estriol methyl ether had a partition coefficient of 1.3 and the radioactivity a partition coefficient of 2.3, thus confirming the small separation observed in the original 250 transfer distribution.

DISCUSSION

The data presented herein demonstrate the ability of three endocrine tissues to carry out the conversion of testosterone to estrogenic steroids. The ability of ovarian tissue to produce estradiol from testosterone has been reported previously (1, 2). Although markedly different in origin and structure, these tissues are all considered capable of producing estrogens *in vivo.*

Stallion testes have been found to contain a very high concentration of estrogenic steroids (16). It is assumed that the estrogens in stallion urine (17, 18, 19) are metabolites of the estrogen produced by the testicular tissue. The experiment presented here indicates that the testicular estrogen may have as its precursor testosterone or a similar nineteen-carbon steroid produced by the testis. Estrogen has also been demonstrated in human testicular tissue, both normal (20) and neoplastic (21), and it is reasonable to suppose that its mode of formation is similar.

The conversion of testosterone to estrogenic steroids by the adrenal carcinoma is a finding consistent with the evidence indicating that such tumors are capable of producing estrogens *in vivo.* The patient in this study had gynecomastia and loss of libido, both signs of overproduction of estrogens. In addition, his urinary excretion of estrogens was markedly elevated prior to the removal of the tumor. Since the conversion of testosterone to estrogenic steroids has been demonstrated in patients lacking adrenals and gonads (22) and since this patient was excreting large amounts of C_{19} steroids, the possibility exists that the urinary estrogens were metabolites produced in non-endocrine tissue from the compounds secreted by the tumor. Another possibility is that the tumor secreted some substance, perhaps a trophic hormone, which stimulated the secretion of estrogens by the unaffected adrenal or the gonads. The demonstration that the tumor tissue was capable of converting testosterone to estrogenic steroids seems to indicate that the enzymes required for the terminal steps in one pathway for the formation of estrogens were present. This, in conjunction with the evidence from the urinary excretion studies that the tumor produced C_{19} steroids, is strong suggestive evidence that a conversion of this type could be responsible for the production of estrogenic steroids. In males, tumors of the adrenal cortex are often associated with feminization (23). Salhanick and Berliner (24) have recently described the isolation of equilenin from such an adrenal tumor. That the normal adrenal produces estrogens is indicated by the isolation by Beall (25) of estrone from beef adrenals.

In a parallel study in which slices of this adrenal tumor were incubated with 50 μC of CH_3 $C^{14}OO$ Na, no measurable radioactivity was found associated with the estrogens isolated,[9] demonstrating that under the conditions used testosterone was a far better precursor than acetate. In the case of the incubation with C^{14} acetate, however, no labeled neutral steroids could be isolated, indicating either that the amount of C^{14} acetate was insufficient or the conditions unsatisfactory for appreciable incorporation of acetate into the steroids.

The third experiment demonstrates the ability of placental tissue to synthesize estrogenic steroids from testosterone and is in accord with the observations of Ryan who demonstrated aromatization of testosterone by human placental microsomes in the presence of reduced triphosphopyridine nucleotide and molecular oxygen (26). The human placenta has been well established as a source of estrogens both by direct isolation (27, 28, 29) and by the effect of its presence on urinary estrogens (30). In addition, dog placenta was shown by Meyer to convert 4-androstene-3,17-dione and 19-hydroxy-4-androstene-3,17-dione to estrone (4).

These experiments together offer further suggestive evidence that testosterone or a similar nineteen carbon steroid is a normal intermediate in the biosynthesis of estrogenic steroids. Although these experiments are not strictly comparable since the incubation conditions were different, the differences in the extents of conversion of testosterone to estrogens are striking. The relatively small conversion of testosterone by the stallion testis is consistent with the fact that the predominant function of testis is to secrete C_{19} steroids. The low conversion by the adrenal tumor fits well into the over-all nature of its secretion as judged by urinary steroids. In this respect the tumor is similar to the stallion testis. Although the effects of the estrogens on the male host were marked, estrogens were quantitatively minor products compared with the C_{19} steroids.

The striking conversion of testosterone to estrogens by the placenta is of particular interest. If testosterone is an intermediate in the synthesis of estrogens by this tissue, it must undergo rapid turnover since there is no evidence suggesting that the placenta releases significant quantities of androgens into the blood stream. The enzymatic reaction limiting the production of estrogen is more likely to be one involved in the synthesis of testosterone rather than in the conversion of testosterone to an estrogenic steroid. It is interesting to consider the possible role of testosterone as a precursor of estrogens in the placenta in the light of other steroids produced by this organ. Progesterone, a well-established hormone of the placenta, has been shown to be oxidized to 17α-hydroxyprogesterone by testicular and ovarian tissue (31, 32, 33, 34). The ability of the placenta to form 17α-hydroxyprogesterone has been indicated by the work of Herrmann (35) who found an increase in urinary excretion of pregnane-3α, 17α, 20α-triol in pregnancy. The conversion of 17α-hydroxyprogesterone, in turn, to C_{19} steroids has been demonstrated in testicular tissues (32), although not in placental tissue. The steps by which placental progesterone may be converted to placental estrogens are, therefore, nearly all established. This interesting relationship between the two classes of steroidal female sex hormones was suggested earlier by Solomon *et al.* (34) with reference to the ovary, and more recently supported by the experiments of Savard *et al.* (36) with the pregnant mare.

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[9] Engel, L. L. and B. Baggett. Unpublished data.

The high activity of the placenta with respect to steroid metabolism is further attested by the presence of as yet unidentified phenolic transformation products of testosterone. Attention is drawn in particular to the polar substance which followed the carrier estriol with such tenacity and which were separated only after a 250 transfer counter-current distribution and methylation. These findings emphasize once more the need for the application of stringent criteria of radiochemical purity before concluding that a radioactive transformation product and an added carrier are identical.

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NOTES AND COMMENTS

THE EFFECTS OF NEUROHYPOPHYSIAL EXTRACTS ON THE TADPOLE OF THE FROG, *HELEIOPORUS EYREI*

ABSTRACT

Heleioporus eyrei tadpoles increase their body weight when injected with Pitocin but not Pitressin. This response takes place at a relatively early stage of development compared to the other species previously tested.

Adult Anura respond to injections of neurohypophysial extracts by increasing their body weight. This is due to an increase in the rate of water uptake through the skin and an antidiuresis (1). This response has not yet been demonstrated in marine and freshwater gill-breathing species (2) with the notable exception of the axolotl (3). The tadpoles of Anura are in an interesting position in that they are a fresh water form that breathes by mean of gills, destined to become a terrestrial species with all the associated problems of water balance. The tadpole of the toad *Bufo bufo* does not respond, by increasing its body weight, to injection of neurohypophysial hormone till it has reached the stage of development where its tail disappears (4). Whether all larval Anura respond at such a relatively late stage of development is not known but it appeared possible that it may develop earlier in a species that will live in drier conditions than *Bufo bufo.*

The tadpoles of the burrowing frog *Heleioporus eyrei* which lives in a large area in southwest Australia were studied.

Injections of Pitressin and Pitocin (Parke, Davis & Co.) were given with an "Agla" micrometer syringe. The skin at the base of the tail was punctured and the needle pushed under the skin to the center of the back where the injection was made. The hormones were given in distilled water (1% of the body weight). The animals ranged in weight from 1.5 to 2.8 gm. and were matched for size and state of development in two groups. Hind limb rudiments were visible but the fore limbs were not due to appear for several weeks. The animals were weighed to 0.1 mg. in a tared weighing bottle containing tap water. During the experiments the animals were kept in tap water at 16 to 18° C and not fed.

With Pitressin 1 U./100 gm. there was a loss in weight over a 3 hr. period of 0.61 gm./100 gm. (S.E. ± 0.92) in 18 animals while with the same dose of Pitocin there was an increase in weight of 4.01 gm./100 gm. (S.E. ± 0.82) in 16 animals. This difference in response is highly significant ($t = 3.696$ $P < 0.001$). Thus these tadpoles show a differential response to Pitocin and Pitressin, the former being active in increasing the body weight as found in adult *H. eyrei* (5). Whether this increase in weight is due to an antidiuresis and/or an increase in water uptake through the skin is unknown but the definitive mesonephric kidney takes up trypan blue which indicates that it is functional (6).

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EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE ADMINISTRATION UPON THE GOLGI APPARATUS IN THE ISLETS OF LANGERHANS IN THE RAT

ABSTRACT

The histology of the islets of Langerhans of the hypophysectomized rat was studied using the state of the Golgi apparatus as a criterion of activity. This seems to be a somewhat more sensitive indicator than the general stains usually employed. Twelve male Long-Evans rats were hypophysectomized at 40 days of age and sacrificed 14 days post-operatively along with a group of 12 normal pair-fed controls, 6 normal *ad libitum*-fed controls and 4 animals fed *ad libitum* and given 2.5 mg. of growth hormone per day intraperitoneally for 4 days. Slides were prepared for demonstration of the specific granules and the Golgi apparatus. In none of the groups were significant changes noted in the granules of either alpha or beta cells, but in the Golgi preparations there was striking atrophy of the beta cell Golgi bodies in the hypophysectomized animals as compared with either the *ad libitum*- or the pair-fed controls. Treatment with growth hormone restored the Golgi apparatus toward normal, but the significance of this observation remains in doubt because the food intake was not restricted. The alpha cell Golgi bodies were not altered in any of the groups.

Since it was observed that administration of large amounts of anterior pituitary extracts would produce permanent diabetes in the dog, considerable work has been devoted to elucidating the normal relationships between the pituitary and the islets of Langerhans. A trophic relationship between the pituitary and the beta cells of the islets similar to that existing between the pituitary and other target organs was postulated by Anselmino and coworkers (1, 2). It has been since confirmed that anterior pituitary extracts (3), and particularly growth hormone (4), will produce islet hyperplasia. Nevertheless, the idea of a trophic relationship has been discarded because hypophysectomy does not result in atrophy of the beta cells similar to that found in the target organs for which such a relationship has been established. Furthermore, there is no evidence indicating that the ability of the pancreas to produce insulin is impaired.

The relationship between the anterior pituitary and the alpha cells of the islets has only recently been explored and is clouded by conflicting evidence. Ferner, *et al.* (5) and Kracht (6) have claimed that hypophysectomy reduces the ratio of alpha to beta cells and that administration of growth hormone restores the normal ratio. Their studies were based upon silver stains for demonstration of the alpha cells, a procedure which has been called into serious question by Volk, Goldner and Frank-Crowley (7). These authors have pointed out the capricious nature of silver preparations and have restudied the islets of hypophysectomized rats using several other stains which they felt would demonstrate the alpha cells more reliably. They were unable to find evidence of atrophy of the alpha cells, nor changes following growth hormone administration except after very prolonged treatment. Under these conditions, the number of alpha cells initially increased and then decreased. Other investigators have found either no change in the alpha cells after growth administration (8) or an increase in activity (9).

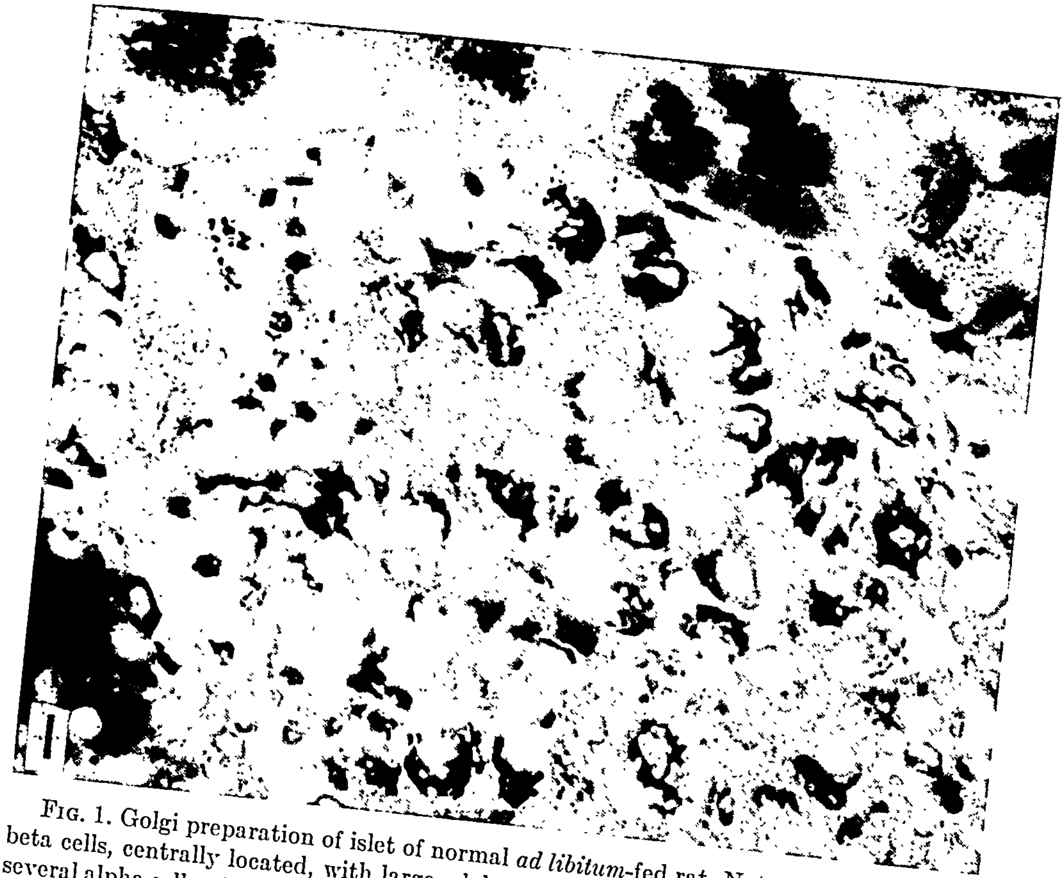

FIG. 1. Golgi preparation of islet of normal *ad libitum*-fed rat. Note preponderance of beta cells, centrally located, with large, elaborate Golgi bodies. Left pole of islet shows several alpha cells with typical clear cytoplasm and small Golgi bodies. ×917.

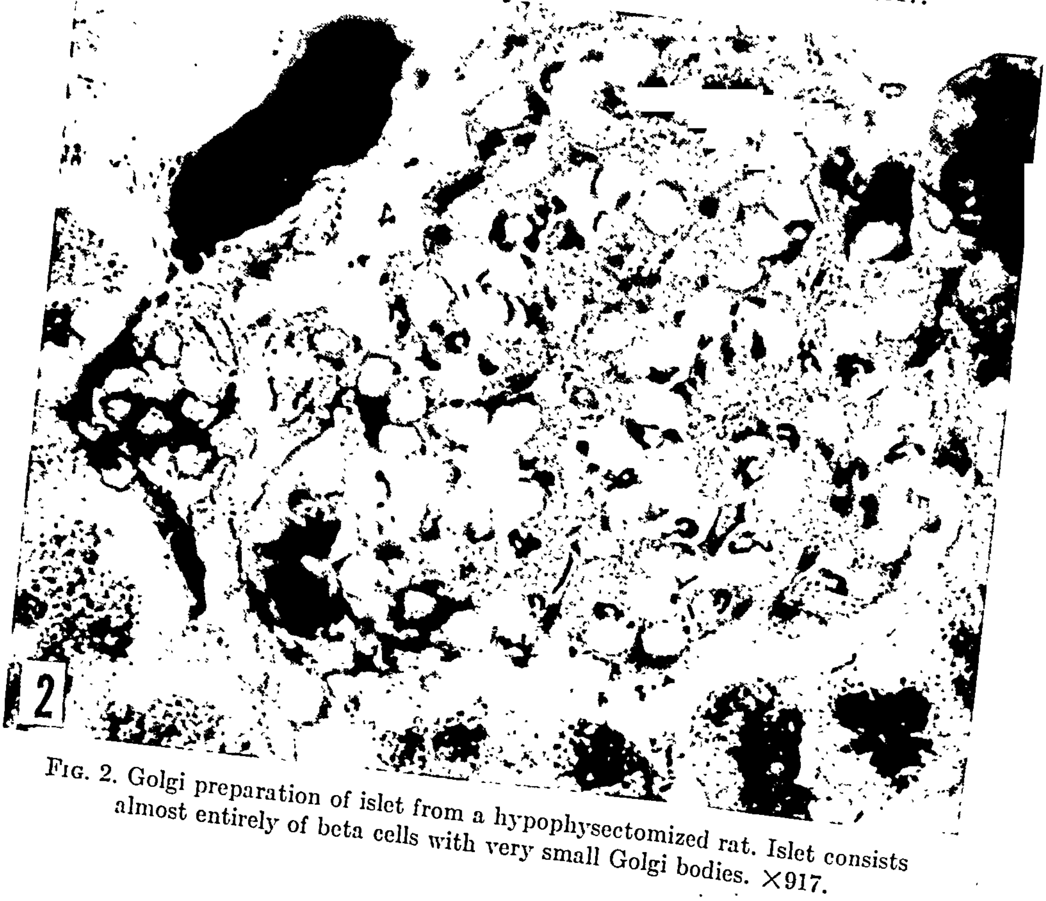

FIG. 2. Golgi preparation of islet from a hypophysectomized rat. Islet consists almost entirely of beta cells with very small Golgi bodies. ×917.

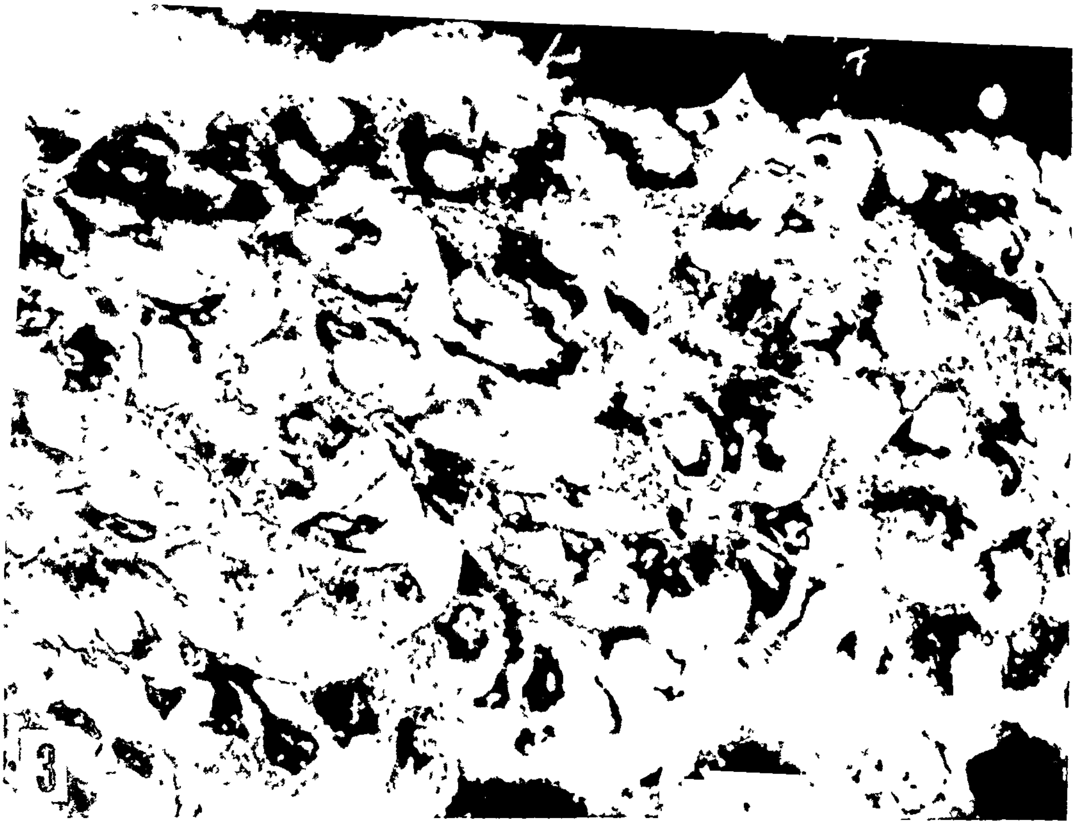

FIG. 3. Golgi preparation of islet from a control rat pair-fed with the animal whose islet is seen in Figure 2. Islet consists mostly of beta cells with much more elaborate Golgi bodies than seen in Figure 2. ×917.

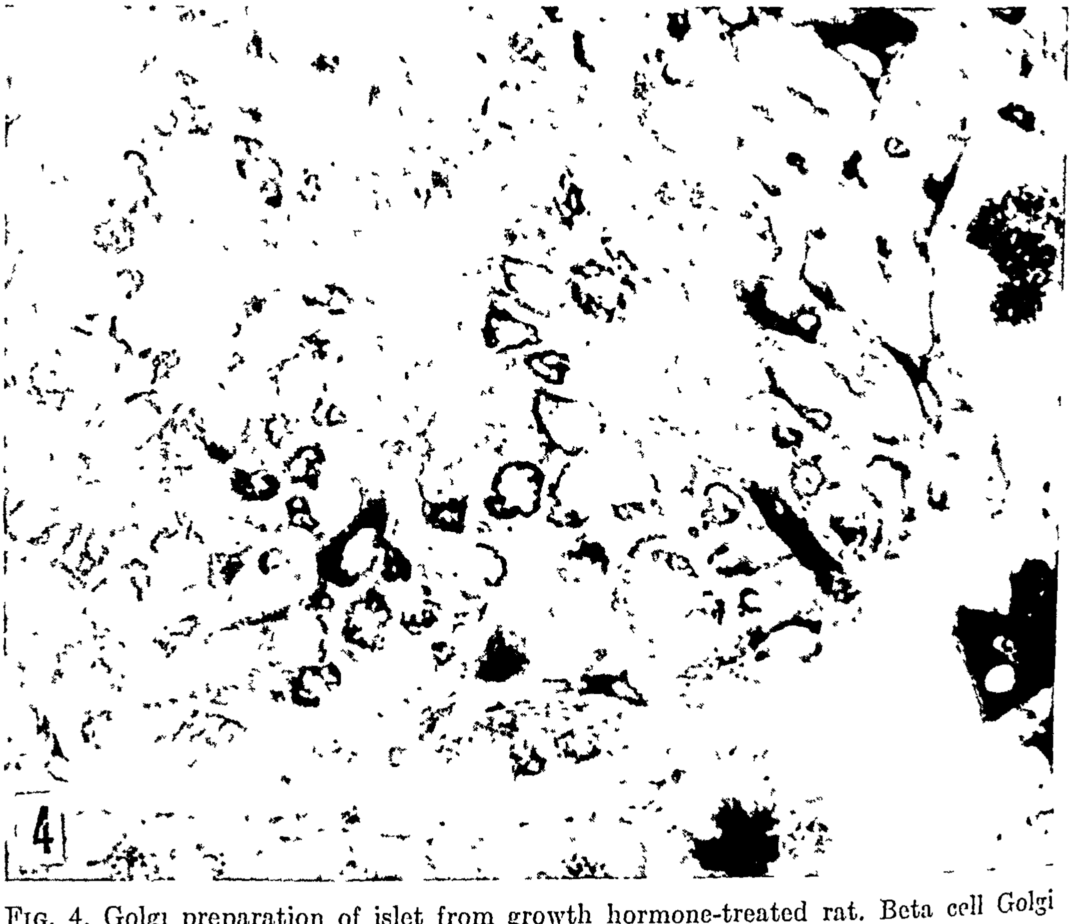

FIG. 4. Golgi preparation of islet from growth hormone-treated rat. Beta cell Golgi bodies are more elaborate than those of hypophysectomized rat in Figure 2. ×917.

Enough uncertainty still remains about the effects of altered levels of pituitary function on the alpha and beta cells that it seemed worthwhile to restudy the islets of rats after hypophysectomy and after growth hormone administration, using as a criterion of activity the state of the Golgi apparatus.

METHODS

Twelve male Long-Evans rats were hypophysectomized by the [illegible]proach at 40 days of age and were then placed in individual cages so that t[illegible] could be measured. Twelve normal rats of the same sex and age were also [illegible] cages at this time and each was pair-fed with one of the hypophysectomized rats. Both groups were sacrificed after 14 days. Completeness of operation was ascertained at autopsy by examination of the operative site using a binocular dissecting microscope. A group of 4 hypophysectomized female rats of 40 days of age was given 2.5 mg. of growth hormone per day intraperitoneally, with food allowed *ad libitum*. These animals were sacrificed after 4 days of treatment together with a group of normal controls also fed *ad libitum*. Other animals treated with growth hormone were restricted to the food intake of hypophysectomized animals, but unfortunately, these animals died, presumably of hypoglycemia, as described by Milman and Russell (10). In all cases, part of the tail of the pancreas was fixed in Bouin's fluid, cut at 4 μ and stained with Gomori aldehyde fuchsin, chromotrope and light green for specific granules, while another part of the tail of the pancreas was fixed in Champy-Kull fixative and the Golgi apparatus impregnated by the Nassonov-Kolatchev method. The growth hormone employed was prepared by the method of Wilhelmi *et al.* (11).

RESULTS

Preparation stained for specific granules. The islets of the normal animals showed well-granulated beta cells and the usual shell of moderately granulated alpha cells. The beta cells in the hypophysectomized animals appeared to be somewhat smaller but contained a normal amount of granular material and looked normal otherwise. The alpha cells were unchanged. The islets of the pair-fed animals were indistinguishable from those of the normal controls.

Golgi preparations. As described previously (12), the two cell types in normal islets showed Golgi bodies of very different appearance. Those of the beta cells were quite large and moderately elaborate while those in the alpha cells were quite small and simple (Fig. 1).

In the beta cells of all of the hypophysectomized animals the Golgi bodies were extremely small and simple (Fig. 2). Those of the alpha cells were apparently unchanged.

The animals which were pair-fed with the hypophysectomized animals showed some reduction in the intricacy of their beta cell Golgi bodies (Fig. 3), but this never approached the degree of change observed in the hypophysectomized animals. The alpha cells appeared to be unchanged when compared with either the hypophysectomized or the normal *ad libitum*-fed controls.

The animals treated with growth hormone showed a definite increase in the size and complexity of the beta cell Golgi bodies and no change whatever in those of the alpha cells (Fig. 4).

DISCUSSION

The basis for using the appearance of the Golgi apparatus as a criterion of the functional state of the islet cells has been discussed in a preceding paper (12). If the interpretation of the changes in the Golgi bodies in the hypophysectomized animal is accepted as indicating a reduction in function, then it is necessary to consider the significance of this change. The explanation could lie along at least two lines. First, the hypophysectomized animals may have had lower blood sugars than did the pair-fed controls, but it is highly unlikely that this was the case, since it is well-established that the hypophysectomized animal allowed food *ad libitum* maintains normal blood glucose levels (13). Second, the diminution in activity might have resulted from removal of one or more pituitary hormones which ordinarily directly stimulate the beta cells. This does not necessarily imply a trophic relationship, however, since the hypophysectomized animal obviously retains a considerable ability to produce insulin. The question of whether or not the beta cells of the hypophysectomized animal are able to respond normally to the stimulus of elevated glucose levels is currently under investigation.

It is not possible at this time to decide which of the pituitary hormones may be involved, since at least ACTH, lactogenic and growth hormone are considered to act as stimuli to the beta cells either directly or indirectly. The observation that growth hormone appeared to restore the Golgi bodies of the beta cells toward normal would tend to favor the possibility that it, at least, is involved. However, it has not yet been possible to separate its effect from changes in food intake, inasmuch as our group of growth-hormone-treated animals on restricted food intake died.

The absence of any change in the appearance of the Golgi apparatus in the alpha cells is another example of their curious constancy of appearance in the face of what are supposed to be stimuli to the release of glucagon.

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ALKALINE PHOSPHATASE ACTIVITY IN THE PITUITARY BODY OF THE RAT

ABSTRACT

The posterior lobe of the pituitary body of the rat has higher alkaline phosphatase activity than the anterior lobe and hypothalamus. This high alkaline phosphatase activity in the posterior lobe is due to a concentration of the enzyme in the capillary walls of the lobe. Alkaline phosphatase in the capillary walls is more active in the posterior lobe than in the anterior and intermediate lobes. The posterior lobe has more densely arranged blood vessels than the two other lobes in the pituitary body. Alkaline phosphatase activity of the posterior lobe in the rat is not changed by dehydration or administration of corticoids. The relation between alkaline phosphatase activity in the posterior lobe and release of hormones into the circulation is discussed.

Recent histochemical studies have shown that there is a correlation between presence of alkaline phosphatase in cells and activity in transport of materials. Capillaries provide a most striking example of this (1). On the other hand, it has been demonstrated that a rapid discharge of antidiuretic hormone from posterior lobe to blood is elicited by stress (2). Therefore, it is likely that in the posterior lobe of the pituitary gland there must be an active movement of hormonal substances from nerve endings to blood vessels. The present experiments were designed to study the relation between the activity of alkaline phosphatase of the posterior lobe and release of hormones from the lobe into blood vessels in the rat. Furthermore, the enzyme activity was compared with that of the anterior lobe and hypothalamus.

MATERIAL AND METHODS

Female adult rats, weighing about 180 gm. were used for the study of characteristics and distribution of the alkaline phosphatase of the tissues employed. For the study of the localization of alkaline phosphatase, the tissues were treated essentially according to the method of Gomori (3). They were fixed in cold acetone for 2 hours. The sections were embedded in paraffin and cut at 6μ. The incubation time was 2 hours in the reaction mixture of pH 9.2–9.8. Control runs without substrate were made in parallel. To assay the enzyme activity, Ohmori's method was employed (4, 5). The tissues of hypothalamus, anterior lobe and posterior lobe including intermediate lobe were weighed on a torsion balance immediately after removal from the body. The homogenates were made to contain a particular concentration of the tissues per 1.0 ml. by adding an appropriate volume of cold de-ionized water. These procedures were carried out at 0–5° C. The homogenate of the posterior lobe included the intermediate lobe. The reaction tube containing 1.0 ml. of ammonium buffer and 1.0 ml. of the substrate, disodium *p*-nitrophenylphosphate (0.05%) was placed for at least 3 minutes in a water bath at 37° C and then 1.0 ml. of the homogenate of tissue was introduced into the tube to start the enzyme action. After the incubation for a fixed length of time at 37° C, with occasional shaking, 1.0 ml. of 20% trichloroacetic acid was added to stop the enzyme action. A 3.0 ml. aliquot was taken from the deproteinized supernatant after centrifugation and added to 3.0 ml. of saturated Na_2CO_3 solution. After some minutes, yellow colored *p*-nitrophenol in this mixture was determined by measuring the optical density at 430 mμ, using an electrophotometer. The activity of phosphatase is given as μg. *p*-nitrophenol liberated in the reaction mixture. To determine the pH optima, the enzyme activity was measured at intervals of 0.2 pH units from 8.4 to 10.4 (ammonium buffer). In these tests the homogenates were the following concentration: hypothalamus, 5.0 mg./cc; anterior lobe of pituitary 1.85 mg./cc; posterior lobe of pituitary 0.66 mg./cc. The incubation time at 37° C was 20, 30 or 40 minutes for hypothalmus, anterior lobe or posterior lobe, respectively. Four similar determinations were made. In another series of experiments, the effect of varying enzyme concentration on phosphatase activity was observed using the following different amounts of the homogenized tissues per cubic centimeter: hypothalmus, 1.0, 3.0 and 6.0 mg.; anterior lobe, 1.0, 1.5 and 3.0 mg.; posterior lobe, 0.4, 0.8 and 1.6 mg. The incubation time was 20 (hypothalamus), 30 (anterior lobe) or 40 minutes (posterior lobe) at 37° C at the optimal pH. The relation of the length of incubation (10, 30 and 40 minutes) to total enzymal activity was tested using tissue homogenates of 5.9 mg. of hypothalamus, 1.33 mg. of anterior lobe, or 0.44 mg. of posterior lobe per 1 ml. at 37° C at the optimal pH. After the separate determination of these enzyme characteristics, the alkaline phosphatase activity of the three tissues was compared under the following conditions in each of 6 animals. The homogenates of hypothalmus, anterior lobe and posterior lobe were prepared to contain 5.0, 1.5 and 0.5 mg. per 1 ml. and the incubation time at 37° C was 20, 40,

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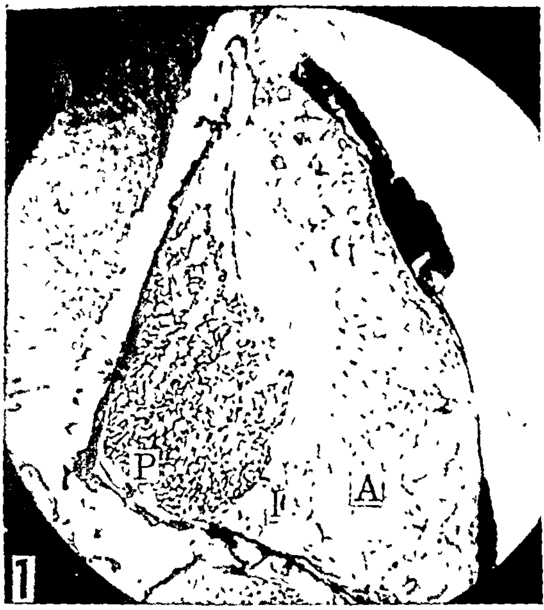
and 40 minutes at the optimal pH, respectively. The relative enzymal activity of the three tissues was calculated from these simultaneous data. Control runs, blanks without added tissue, were always made in parallel with the experimental ones.

In dehydration experiments, two groups of littermate rats were used, with 6 adult females in each group. The body weights were between 160 and 195 gm. Three females of one litter, and three of another, did not receive water for 7 to 11 days, but were allowed to take food *ad libitum*. The 6 remaining rats were given food and water freely and served as controls. The alkaline phosphatase activity of the posterior lobe of each animal was determined at the optimal pH and 37° C. The incubation time was 40 minutes. The concentration of the tissue was 0.4–0.5 mg. per 1.0 ml. of the homogenate. In the next experiment, the effects of desoxycorticosterone acetate (DCA) and cortisone acetate on the activity of the enzyme were studied. Four adult female rats, weighing about 130 gm., received 5 injections of 5 mg. of DCA. The injections were made every other day and the animals were killed on the day after the last injection. Five other female rats, weighing about 130 gm. were given 5 mg. of cortisone acetate and treated in the same way as in the DCA experiment. Six untreated female rats served as controls. The alkaline phosphatase activity of the posterior lobe of each animal was assayed.

RESULTS

Deposits of cobalt sulfide, indicating alkaline phosphatase activity, were most dense in the capillary walls of posterior lobe. In fact, the reaction of the capillary walls was higher in the posterior lobe than in the anterior and intermediate lobes of pituitary body (Figs. 1 and 2). The figures show also more densely arranged blood vessels in the posterior lobe than in the anterior and intermediate lobes. There is at present no agreement regarding the presence of alkaline phosphatase in nuclei as revealed by the Gomori's method, however, using this technique, deposits of cobalt sulfide were found to be heavier in the nuclei of anterior lobe cells than in those of the posterior and intermediate lobes, and were not found in cytoplasm of the cells of three pituitary lobes, under the experimental conditions used.

The activity optimum of the phosphatase was around pH 10.0 for the hypothalamus, anterior lobe and posterior lobe including intermediate lobe. The activity of the enzyme in the three tissues was linear with respect to the enzyme concentration used, and had a linear relation to the incubation time between 10 and 40 minutes at pH 10.0 and 37° C.



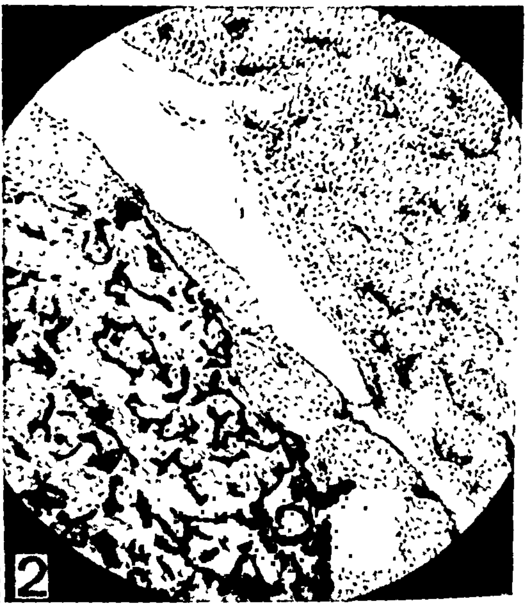


FIG. 1. Alkaline phosphatase in pituitary body of rat. Note strong reaction in capillary walls in posterior lobe. P, posterior lobe; I, intermediate lobe; A, anterior lobe, ×30.

FIG. 2. Higher magnification of Fig. 1. ×95.

The amounts of *p*-nitrophenol liberated in a period of 40 minutes by 1.0 mg. of each tissue at pH 10.0 and 37° C were 4.7 ±0.40 µg. by hypothalamus, 2.0 ±0.25 µg. by anterior lobe and 17.1 ±0.76 µg. by posterior lobe. The figures represent the averages of 6 animals, and the respective standard errors. The alkaline phosphatase activity was higher in the posterior lobe than in the hypothalamus or anterior lobe. By neither dehydration, or administration of DCA or cortisone acetate was the enzymal activity of posterior lobe tissue changed (Table 1).

TABLE 1. EFFECTS OF DEHYDRATION AND CORTICOIDS ON ACTIVITY OF ALKALINE PHOSPHATASE IN POSTERIOR LOBE OF PITUITARY BODY OF RAT (pH 10.0, 37°C.)

Treatments	Number of animals	*p*-Nitrophenol (µg.) liberated in 40 minutes per 1 mg. of tissue
Dehydrated animals	6	19.8 ±0.60*
Controls	6	18.7 ±1.07
Animals injected with DCA	4	22.7 ±2.45
Animals injected with cortisone acetate	5	21.4 ±1.93
Controls	6	22.1 ±1.49

* Standard error.

DISCUSSION

The high activity of alkaline phosphatase of the posterior lobe may be due chiefly to the enzyme activity in capillary walls since the histochemical study showed a high concentration of the enzyme in these sites. Considering the results of his stalk-section experiments, and the observations made by several other workers, Barrnett suggested that the blood vessels of the posterior lobe are permeable to large molecules such as vital dye proteinates or silver salts combined with protein, whereas brain capillaries are generally impermeable (6).

From these observations, it may be supposed that the characteristic high alkaline phosphatase activity of the walls of blood vessels may have a role in the permeability of the blood vessels to neurosecretory substances or posterior lobe hormones and to such proteinates as those discussed by Barrnett. But the activity of alkaline phosphatase in the posterior lobe of the rat was not changed by the following treatments, which may accelerate the release of posterior lobe hormones: dehydration or administration of either DCA or cortisone acetate. It would seem, therefore, that the activity of the enzyme in the capillary walls of the posterior lobe would not be a limiting factor in controlling the rate of release of neurohypophyseal hormones into the blood. It is not clear what the role of abundant alkaline phosphatase is in the capillary walls of posterior lobe. Rich vascularity in the posterior lobe has also been observed by Ferrer (7) using India ink. This vascularity may assure the rapid movement of the hormones from posterior lobe into the blood circulation.

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IMPROVED INTRAVENOUS GLUCOSE TOLERANCE IN THYROIDECTOMIZED OR HYPOPHYSECTOMIZED RATS TREATED WITH TRIIODOTHYRONINE[1]

ABSTRACT

Treatment with triiodothyronine improved or normalized the diminished intravenous glucose tolerance of thyroidectomized or hypophysectomized male rats.

Impaired disposal of intravenously injected glucose has been observed both in thyroidectomized (1, 2) and in hypophysectomized (3) rats. In the present study attempts to alleviate this diminished glucose tolerance by means of treatment with triiodothyronine have proved successful.

MATERIAL AND METHODS

Male Holtzman rats weighing approximately 200 gm. were surgically thyroidectomized under ether anesthesia. All rats that were used in this study had no macroscopically recognizable thyroid remnants at the time of necropsy, and were severely hypothyroid, as indicated by a reduced growth rate as well as (in the animals not treated with thyroid hormone) by the characteristic gelatinous appearance of the anterior hypophysial lobe (4) which is due to degranulation of the acidophils. Unoperated controls were either selected to match the final weight of the thyroidectomized animals (220–240 gm.) or were pair-fed with them. Hypophysectomized male Sprague-Dawley rats weighing 180–200 gm. were obtained 8 days after the operation from the Hormone Assay Laboratories, Chicago, Ill. All rats used had no hypophysial fragments detectable by inspection of the sella turcica and markedly atrophic testes. L-3,5,3′-triiodothyronine sodium (T_3) was dissolved in water made alkaline with NaOH and injected once a day subcutaneously in a volume of 1 ml. Dosages and schedules are given in Table 1. One group of thyroidectomized rats was subcutaneously injected with a single dose (1 mg./100 gm.) of somatotrophin (STH)[2] in a mildly alkaline solution 12 hours before the glucose tolerance test. All rats were fed Rockland diet and fasted 24 hours before the performance of the glucose tolerance test. Glucose (100 mg./100 gm. in 0.26 ml. of aqueous solution) was injected into the saphenous vein without anesthesia. One tenth ml. blood samples were obtained from the tail vein before and (at the intervals indicated in Table 1) after the injection. Blood sugar levels were determined by the Somogyi-Nelson method.

RESULTS

Table 1 summarizes the findings obtained. Thyroidectomized rats had variable fasting blood sugar levels which averaged higher than those of controls fed *ad libitum* before the final fast (Groups Ia+b vs. IV). Their glucose tolerance was clearly impaired in comparison with that of *ad libitum* fed-fasted or pair-fed-fasted (Group V) controls. This confirms earlier observations (1, 2). The fasting hyperglycemia and unimpaired glucose tolerance of the controls on the restricted regimen is also in agreement with

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[2] STH was kindly provided by Dr. A. E. Wilhelmi on behalf of the National Institutes of Health.

TABLE 1. INTRAVENOUS GLUCOSE TOLERANCE OF THYROIDECTOMIZED OR HYPOPHYSECTOMIZED RATS

Group	No. rats	Operation	Test performed days postop.	Treatment	Dose/day	Treatment before test	Initial blood sugar	Blood sugar change†, minutes after i.v. glucose			
								10	30	60	120
								mg.%			
Ia(1)**+b	8	Thyrex	34–35	—	—	—	95 ±8*	+198 ±8	+174 ±8§	+128 ±11§	+ 58 ±7§
II	8	Thyrex	33–38	T_3	20 μg.	last 4 days	117 ±2	+142 ±4	+ 93 ±8	+ 40 ±5	− 7 ±5
III	4	Thyrex	35	STH	1 mg./100 gm.‡	12 hours	79 ±4	+211 ±3§	+202 ±4§	+154 ±7§	+ 57 ±7§
IV	8	—	—	—	—	—	78 ±2	+159 ±9	+104 ±10	+ 44 ±5	0 ±2
Ia(1)	3	Thyrex	35	—	—	—	74 ±2	+205 ±9	+189 ±9	+159 ±15	+117 ±34
Ia(2)	3	Thyrex	45	T_3	2 μg.	last 10 days	92 ±10	+194 ±3	+175 ±8	+121 ±12	+ 1 ±11
Ib	5	Thyrex	34	—	—	—	108 ±6	+193 ±13	+165 ±9§	+110 ±7§	+ 23 ±6
V	5	—	—	pair-fed with Ib	—	—	89 ±1	+170 ±3	+104 ±8	+ 39 ±4	− 1 ±4
VI	7	Hypex	24	—	—	—	60 ±2	+155 ±8	+125 ±8	+ 80 ±11	+ 22 ±9
VII	8	Hypex	24	T_3	2 μg.	last 10 days	65 ±3	+134 ±7	+ 80 ±7§	+ 36 ±7§	− 3 ±4

* Mean ±standard error.
† Compared with initial blood sugar.
‡ Single dose.
§ *Difference from control* significant at 1% level (control groups: IV, V, VI).
** Ia (1) and (2) refers to two determinations on the same group of rats.

previous findings (5). STH failed to alleviate the glucose intolerance of thyroidectomized rats (Group III). Treatment of 3 rats (Group Ia) with 2 μg. of T_3 daily for 10 days led to some elevation of fasting blood sugar levels and a definite improvement in glucose tolerance. Twenty μg. of T_3 given daily for 4 days caused uniform fasting hyperglycemia[3] and normalization of glucose tolerance (Group II). Hypophysectomized rats (Group VI) displayed the expected fasting hypoglycemia and the somewhat diminished glucose tolerance[4] previously described (3). The fasting blood sugar level was not significantly affected but the glucose tolerance was returned to normal by the daily injection of 2 μg. of T_3 for 10 days (Group VII).

DISCUSSION

The decreased glucose tolerance seen both in thyroidectomized and, to a lesser extent, in hypophysectomized rats could be due to hypothyroidism or conceivably to STH deficiency. STH is generally believed to be produced by the pituitary acidophils. The degranulated acidophils of thyroidectomized rats appear to contain and secrete subnormal amounts of STH (6, 7). STH increases the rate of removal of blood glucose in dogs (8) and in rats (9). Treatment with thyroid hormone not only abolishes the hypothyroidism of thyroidectomized rats but also regranulates their pituitary acidophils (3) and hence presumably restores STH secretion. However, under the conditions of our experiment STH failed to influence the poor glucose tolerance of thyroidectomized rats. Further, the improved glucose tolerance which T_3 brought about in hypophysectomized rats obviously could not be ascribed to any effect mediated through the hypophysis. It is therefore simplest to postulate that the decreased glucose tolerance which characterizes both the thyroidectomized and the hypophysectomized state is due to the hypothyroidism common to both conditions. It may be caused by thyroid hormone deficiency *per se,* or by concomitant changes such as the lowered metabolic rate. Further studies will also have to show whether any specific pathway of disposal of excess glucose is preferentially or exclusively affected, and if so, whether thyroid hormone restores normal glucose tolerance by improving the removal of blood glucose along the same pathway.

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[3] The most likely reason for the hyperglycemia due to T_3 is enhanced gluconeogenesis from protein. The paradox of fasting hyperglycemia and normal glucose tolerance may indicate that different mechanisms are involved in the disposal of a sudden excess of exogenous glucose and the maintenance of normal blood sugar levels in the face of a sustained increase of glucose output by the liver.

[4] Cf. blood sugar excess at 60 minutes after glucose injection in group VI, Table 1, with values in untreated groups IV and V ($p<0.01$).

[5] *Medical student summer research fellow.*

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THE ACTIVITY OF SYNTHETIC ISOESTRONES IN PYRIDINE NUCLEOTIDE TRANSHYDROGENATION[1]

ABSTRACT

Synthetic isoestrones were examined for the capacity to mediate pyridine nulceotide transhydrogenation or to act as substrates for 17β hydroxy-steroid dehydrogenase. 8-isoestrone was active in both systems. The other isoestrones tested showed neither activity.

Previous studies have indicated that certain steroids have the property of accelerating transhydrogenation from a catalytic amount of TPNH to DPN in the presence of placental homogenate (1, 2). The structural requirements for striking stimulation of this system are the presence of an aromatic ring A and a 17B hydroxyl or 17 keto group (3, 4). The existence of synthetic isomers of estrone (5) permitted study of other steric requirements.

METHODS

The preparation of the place [illegible] assay system for measuring steroid coenzyme activity in terms of [illegible] production from isocitrate by placental homogenate have been d[illegible] surement of 17β hydroxy-steroid dehydrogenase at low steroid levels it was necessary to purify the placer[illegible] tially, by precipitating the activity at 50% saturation with ammonium [illegible] the redissolved protein against 0.01 *N* phosphate buffer, pH 7.0.

The action of the 17β hydroxy-steroid dehydrogenase (6) was measured by the direct fluorimetric estimation of DPNH in an Aminco-Bowman Spectrofluorimeter. The substrate mixture consisted of phosphate buffer 0.006 *M*, pH 6.8, $MgCl_2$ 0.006 *M*, Versene 0.001 *M*, 0.01 ml. of propylene glycol containing 0.003 μ-moles of the appropriate steroid, and 50 μg. enzyme in a total volume of 1.0 ml. The reaction was started by addition of 0.005 μ-moles of DPNH to the substrate mixture at room temperature.

RESULTS

Table 1 shows that of the 5 synthetic isoestrones[2] tested only dl-8-isoestrone showed significant ability to increase the rate of transhydrogenation from TPNH to DPN as shown by increased α-ketoglutarate formation. In the 17β hydroxy-steroid dehydro-

Table 1. Effect of isoestrones on placental transhydrogenase

Compound	$3.6\times10^{-7}M$	$3.6\times10^{-6}M$	$3.6\times10^{-5}M$
estradiol-17β	0.116	0.122	0.126
estrone	0.076	0.117	0.120
dl-8-isoestrone	0.044	0.112	0.097
dl-9-iso-13-isoestrone	0.001	0.003	0.004
dl-13-isoestrone	0.0	0.0	0.0
dl-9-isoestrone	0.0	0.0	0.0
dl-14-isoestrone	0.0	0.0	0.0

Each value represents the mean obtained from three experiments. Triplicate determinations in each experiment agreed within 5%. The results are expressed in micromoles of ketoglutarate produced in 1 hour at 30° due to the steroid.

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[1] This work was supported by United States Public Health Service Grant CY-3225-C1.

[2] The synthetic isoestrones used in this study were the very kind gift of Professor W. S. Johnson, Department of Chemistry, University of Wisconsin.

TABLE 2. INHIBITION OF ESTRADIOL INDUCED TRANSHYDROGENATION

Compound	$3.6 \times 10^{-5}M$	$3.0 \times 10^{-6}M$
dl-13-isoestrone	84	23
dl-9-iso-13-isoestrone	56.5	14
dl-9-isoestrone	62	14
dl-14-isoestrone	77.3	16.5

The results are expressed as the per cent inhibition of ketoglutarate production by $3.6 \times 10^{-6}M$ estradiol-17β in 1 hour at 30°C.

genase system the only isoestrone with activity was dl-8-isoestrone. This substance was reduced at the same rate as estrone, 1.8×10^{-4} μ-moles per minute.

The four inactive isoestrones were found to inhibit estradiol-17β potentiation of α-ketoglutarate by the placental pyridine nucleotide transhydrogenation system (Table 2).

DISCUSSION

The activity of 8-isoestrone and inactivity of the other isoestrones at the level tested in both the 17β hydroxy-steroid dehydrogenase and pyridine nucleotide transhydrogenation systems is consistent with the mechanism proposed by Talalay and Williams-Ashman that transhydrogenation is effected by the dehydrogenase capable of reaction with both pyridine nucleotides (2). The oxidation of 8-isoestrone at higher substrate levels by 17β hydroxy-steroid dehydrogenase has been observed by Langer (7). The data presented are consistent with the need for a reactant to approach the steroid from the alpha side along a flat portion of the steroid molecule comprising rings C and D. Munck, Scott, and Engel concluded that this portion of the molecule was involved in purine binding (8). Marcus and Talalay studied the substrate specificity of a 17β hydroxy-steroid dehydrogenase-DPN and found that the kinetic data were consistent with interaction of the enzyme system with the α side of the steroid molecule and positions 3 and 17 (9).

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[3] United States Public Health Postgraduate Fellow.

The Endocrine Society

1959 PROGRAM OF THE MEETING OF THE ENDOCRINE SOCIETY IN ATLANTIC CITY

THURSDAY, JUNE 4, 1959

8:00 A.M.—Registration—

I. 9:00 A.M.—THYROID

Carolina Room—Chalfonte

Chairmen: *J. E. Rall and W. Parson*

1. The Effect of Trypan Blue on Thyroid Function in the Rat.
   Takashi Yamada (Introduced by Monte A. Greer).
2. The Effect of Excessive Iodide on the Thyroid of the Rat.
   Valerie A. Galton and *Rosalind Pitt-Rivers.* (Introduced by Sidney Ingbar).
3. Comparison of in Vivo and in Vitro Tissue Effects of Triiodothyronine.
   Roger L. Greif.
4. The Effect of Triiodothyropropionic Acid on Serum Cholesterol and Oxygen Consumption in Myxedema and Euthyroidism.
   Robert D. Leeper (by invitation), *Richard S. Benua, Soichi Kumacka* (by invitation), *Jacob L. Brener* (by invitation) and *Rulon W. Rawson.*
5. Respiratory Stimulation in Young Dogfish by Thyroid Hormone.
   Aubrey Gorbman and *A. W. Pritchard* (by invitation). (Invited paper—20 minutes.)

INTERMISSION

6. Idiopathic Familial Elevation of Thyroxine Binding Protein.
   William H. Beierwaltes.
7. Metabolism of Thyroxine by Human Tissue Slices.
   Edwin C. Albright and *Frank C. Larson* (by invitation).
8. Metabolic Regulation of Organic Iodinations in Thyroid Homogenates.
   George C. Schussler (by invitation) and *Sidney H. Ingbar.*
9. Protein Iodination in Sheep Thyroid Mitochondrial-Microsomal Preparations.
   Leslie J. DeGroot and *Evelyn Carvalho* (introduced by John B. Stanbury).
10. Persistent Thyroid Function After Hypophysectomy.
    O. H. Pearson, A. G. Pazianos (by invitation), *E. Greenberg* (by invitation), and *R. S. Benua.*
11. Effect of Stimulation of the Cervical Sympathetic Nerve on the Thyroidal Release of I^{131}-Labeled Hormones.
    Shiro Iino, Kazuo Shizume and *Shigeo Okinaka* (introduced by Monte A. Greer).

II. 2:00 P.M.—ADRENAL CORTICAL SYNDROMES—STEROID METABOLISM

Carolina Room—Chalfonte

Chairmen: *J. McArthur and A. Bongiovanni*

12. 6 Beta Hydroxylation of Cortisol, a Quantitatively Important Pathway of Metabolism in the Human Newborn.
    E. Colle (by invitation), *R. A. Ulstrom, J. Burley* (by invitation) and *R. Gunville* (by invitation).

13. The Urinary Excretion of Three C-21 Steroids in the Adrenogenital Syndrome.
Alfred M. Bongiovanni, Walter R. Eberlein, and *J. Darrel Smith* (by invitation).

14. The Renal Handling of 17-Hydroxycorticosteroids.
R. O. Morgan (by invitation), *A. J. Blair* (by invitation) and *J. C. Beck.*

15. The Influence of ACTH and Hypophysectomy on the Steroid Conjugating Capacity of the Liver.
David L. Berliner (by invitation), *Charles J. Nabors, Jr.* and *Thomas F. Dougherty.*

16. Direct and Indirect Estimation of Pituitary ACTH Reserves in Man Following Steroid Therapy
Donald A. Holub (by invitation), *Julian I. Kitay, Andrew G. Frantz* (by invitation) and *Joseph W. Jailer.*

17. ACTH Suppression Tests in Patients with Cushing's Syndrome.
Grant W. Liddle.

INTERMISSION

18. Urinary Steroid Metabolites in Hypophysectomized Women During Adrenal Insufficiency and Initial Phases of ACTH Stimulation.
Hildegard Wilson, Mortimer B. Lipsett, and *Lillian C. Butler* (by invitation).

19. Suppression of Urinary 17-Ketosteroids in Virilized Women by Estrogen Therapy.
Raymond C. Mellinger and *Richmond W. Smith, Jr.*

20. Transcortin: Characteristics and Levels in Various Conditions.
Avery A. Sandberg and *W. Roy Slaunwhite, Jr.*

21. Studies on the Mechanism of Elevation of Plasma 17-Hydroxycorticosteroids Induced by Estrogen Administration in Man
Eleanor Z. Wallace and *Anne C. Carter.*

22. Endocrine Effects of Methylenedianiline.
William W. Tullner.

23. Salt Losing Hormone in Hyperkalemic Familial Periodic Paralysis.
R. Klein.

III. 2:00 P.M.—PARATHYROIDS: PITUITARY ADRENAL SYSTEM

Viking Room—Haddon Hall

Chairmen: *G. D. Whedon and D. Nelson*

24. Isolation of Parathyroid Hormone Following Extraction with Phenol.
G. D. Aurbach.

25. Purification of Parathormone B by Countercurrent Distribution.
Howard Rasmussen.

26. Activation of Parathyroid Hormone by Cysteine.
Alexander D. Kenny.

27. Effect of Parathyroid Extract on Bone Matrix and Seromucoid.
William P. Deiss and *Leila B. Holmes* (by invitation).

28. Further Evidence for a Dual Effect of Parathyroid Hormone.
John J. Canary (by invitation), *H. Allan Bloomer* (by invitation), *Gabriel G. Carreon* (by invitation) and *Laurence H. Kyle.*

29. ACTH Secretion in Rats with Hypothalamic Lesions.
A. Brodish.

INTERMISSION

30. Limbic System Influences on the Pituitary-Adrenal Cortical System.
John W. Mason, Walle J. H. Nauta (by invitation), *Joseph V. Brady* (by invitation), and *James A. Robinson* (by invitation).

31. Effect of Diencephalic Stimulation on Adrenocortical and Adrenal Medullary Secretion in the Dog.
William H. Ganong and *Alan Goldfien.*
32. Adrenal Cortical and Medullary Responses to Trauma in Dogs with Isolated Pituitaries.
Richard H. Egdahl (introduced by D. M. Hume).
33. The Early and Late Effects of Hypothalamic Destruction or Removal on Pituitary-Adrenal Activity.
David M. Hume and *Benjamin F. Jackson* (by invitation).
34. Ion-Exchange Chromatography of Pituitary Preparations.
Andrew V. Schally (by invitation) and *Roger Guillemin.*
35. ACTH Releasing Factor Active in the Guinea Pig Test.
M. Sideman (by invitation) and H. Sobel.

FRIDAY, JUNE 5, 1959

8:30 A.M.—Registration—

IV. 9:00 A.M.—ANTERIOR PITUITARY

Carolina Room—Chalfonte

Chairmen: *E. Knobil and F. L. Engel*

36. The Cells of the Human Adenohypophysis in Adrenal Cortical and Thyroid Disorders.
Calvin Ezrin and *Heidi Swanson* (introduced by R. F. Farquharson).
37. A Method for the Quantitative Biological Assay of ACTH in Blood.
Paul L. Munson, Wolfgang Toepel (by invitation) and *Mindel C. Sheps.*
38. Increase in Sugar Permeability in the Rat Adrenal Produced by ACTH.
J. Eichhorn, I. D. K. Halkerston, M. Feinstein, amd *O. Hechter* (by invitation).
39. Contrasting Requirements for the Lipolytic Action of Corticotropin and Epinephrine on Adipose Tissue in Vitro.
J. Earle White (by invitation), *Enrique Lopez* (by invitation) and *Frank L. Engel.*
40. Evidence for Species Specificity of Human, Beef and Rat ACTH Demonstrated by Assay in Vitro.
Julian I. Kitay, Donald A. Holub (by invitation) and *Joseph W. Jailer.*
41. A Comparative Immunological Investigation of Pituitary Growth Hormone from Various Species.
T. Hayashida (by invitation) and *C. H. Li.*

INTERMISSION

42. Growth-Stimulating and Other Effects of Human Growth Hormone (Li) in a Pituitary Dwarf.
Roberto F. Escamilla, John J. Hutchings (by invitation), *William C. Deamer* (by invitation) and *Choh Hao Li.*
43. Endocrine Function in Acromegaly.
George J. Hamwi and *Thomas G. Skillman* (by invitation).
44. The Influence of Hypophysectomy and Growth Hormone on Oxidation of Palmitate-1-C^{14} by the Rat.
M. J. Franklin (by invitation) and *E. Knobil.*
45. Unesterified Fatty Acid Oxidation Rates in Acromegaly, Myxedema, Hyperthyroidism and Diabetes.
Josiah Brown and *Leslie R. Bennett* (by invitation).
46. Evidence for Direct Action of Growth Hormone (GH) on Red Cell Formation.
Albert S. Gordon, Burton S. Dornfest (by invitation) and *Anthony Crusco.*

47. Factors Involved in Production of Suspensions of Free Cells Derived from Hypophyses of Rats.
Griff T. Ross (by invitation) and *Robert C. Bahn.*

1:30 P.M.—ANNUAL BUSINESS MEETING, Carolina Room, Chalfonte

V. 2:30 P.M.—ESTROGENS AND ANDROGENS

Carolina Room, Chalfonte

Chairmen: *R. Huseby and L. L. Engel*

48. The Isolation and Identification of Estrogens from Peripheral Blood with a Practical Method of Analysis.
Georg W. Oertel (by invitation), *Charles D. West,* and *Kristen B. Eik-Nes.*
49. "Free Estriol" in Blood Plasma.
Joseph C. Touchstone and *John W. Greene, Jr.* (by invitation).
50. Effect of Chorionic Gonadotropin (HCG) on Conversion of Estradiol to Estriol by Perfused Human Placenta.
Philip Troen.
51. Physiologic and Biochemical Differences between the Estrogen-Sensitive Transhydrogenase and Estradiol Dehydrogenase.
C. A. Villee, D. D. Hagerman (by invitation) and *J. M. Spencer* (by invitation).
52. The Inhibition of Placental Steroid Mediated Transhydrogenase by 2′ Adenylic Acid.
Nina Hollander (by invitation), *Joseph D. Brown* (by invitation) and *Vincent P. Hollander.*
53. Adrenal Estrogens: In Vivo Conversion of Cortisone to Phenolic Estrogens.
Edward Chang (by invitation) and *Thomas L. Dao.*

INTERMISSION

54. Estrogen Synthesis in a Male Patient with a Feminizing Adrenocortical Carcinoma and Hypertension.
C. D. West, L. F. Kumagai (by invitation), *E. L. Simons* (by invitation) and *P. E. Wall* (by invitation).
55. The Effect of Estrogen on Steroid Levels and Urine.
W. L. Harrmann, I. Schindl (by invitation), and *P. K. Bondy.*
56. The Conversion of Progesterone-4-C^{14} to 21-Hydroxylated Steroids by Normal Rat Testis Stimulated with Human Chorionic Gonadotropin.
Hernan F. Acevedo (by invitation), *Oscar V. Dominguez* and *Leo T. Samuels.*
57. Steroid 21-Hydroxylase in Mouse Testicular'Interstitial Cell Tumors.
Oscar V. Dominguez, Robert A. Husby and *Leo T. Samuels.*
58. Studies of Structure-Function Relationships of Steroids: Anabolic Potency of Some Testosterone Analogs.
Herbert A. Burke, Jr. (by invitation) and *Grant W. Liddle.*
59. Influence of Androgen on Prostatic Protein Synthesis.
Wells E. Farnsworth (introduced by Grosvenor W. Bissell).

VI. 2:30 P.M.—PITUITARY HORMONES—NEUROENDOCRINE MECHANISM

Viking Room, Haddon Hall

Chairmen: *J. Everett and A. Gorbman*

THOSE REGISTERED FOR THE MEETING OF THE AMERICAN DIABETES ASSOCIATION WILL BE ADMITTED TO THIS MEETING

60. Lithosperm Inhibition of Blood Pressure Depressor Effect of Oxytocin in the Fowl.

W. R. Breneman, Marvin Carmack, Daniel Overack and *Richard Shaw* (introduced by Aubrey Gorbman).

61. PLASMA AND PITUITARY LH: DETECTION AND QUANTITATIVE BIOASSAY IN THE RAT FOLLOWING GONADECTOMY.
Albert Parlow (introduced by R. O. Creep).

62. USE OF THE RED EFT DIAMICTYLUS VIRIDESCENS IN THE ASSAY OF PROLACTIN.
William C. Grant, Jr. (introduced by Aubrey Gorbman).

63. EFFECTS OF CAPTIVITY ON PRODUCTION OF GONADOTROPHINS IN PINTAIL AND BLACK DUCKS AND RESPONSE TO EXOGENOUS HORMONES.
R. E. Phillips and *A. van Tienhoven* (introduced by S. L. Leonard).

64. OVULATION IN THE DOMESTIC FOWL.
R. D. Fraps. (Invited paper, 20 minutes).

INTERMISSION

65. OVULATION AFTER STIMULATING THE RAT HYPOTHALAMUS LATE IN DIESTRUS.
J. W. Everett, R. L. Riley (by invitation) and *C. D. Christian* (by invitation).

66. DEVELOPMENT OF PERMANENT ABNORMAL CYCLES OF BEHAVIOR IN RATS AFTER EXPERIMENTAL PITUITARY DAMAGE.
Curt P. Richter.

67. HYPOTHALAMUS AND GROWTH.
S. Reichlin.

68. TSH "REBOUND PHENOMENON" IN RATS AFTER GOITROGEN WITHDRAWAL.
S. A. D'Angelo.

69. PITUITARY HORMONE PRODUCTION AND RELEASE IN THE THYROIDECTOMIZED RAT AFTER THYROXIN ADMINISTRATION.
A. N. Contopoulos, M. E. Simpson (by invitation) and *A. A. Koneff* (by invitation).

70. SUBCORTICAL REGIONS OF THE PRIMATE BRAIN REGULATING RELEASE OF VASOPRESSIN FROM THE NEUROHYPOPHYSIS.
Joseph F. Dingman, Eduardo Gaitan (by invitation), *Akira Arimura* (by invitation) and *Robert G. Heath* (by invitation).

VII. ANNUAL DINNER, Haddon Hall Hotel

E. Perry McCullagh, President, presiding

6:30 P.M.—Cocktails, The English Lounge
Sponsored by the donors of the Awards of the Society:
Ciba, Schering, Ayerst, Upjohn and Squibb. Attendance limited to holders of dinner tickets.

7:30 P.M.—Dinner—The Vernon Room
Presentation of the 1959 Awards of the Society by Charles Kochakian:
The Fred Conrad Koch Award
The Ciba Award
Ayerst Fellowship
Schering Scholar
Upjohn Scholar
President's Address: Dr. McCullagh

SATURDAY, JUNE 6, 1959

8:30 A.M.—Registration—Stair Hall, Haddon Hall Hotel

9:00 A.M.—**OPENING SCIENTIFIC SESSION OF THE AMERICAN DIABETES ASSOCIATION WILL BE HELD AT THE HADDON HALL HOTEL IN THE VERNON ROOM. ENDOCRINE SOCIETY BADGE WILL ADMIT YOU TO THIS MEETING.**

VIII. 9:00 A.M.—ADRENAL GLAND

Carolina Room, Chalfonte

Chairmen: *M. Grumbach and F. C. Bartter*

71. Stimulatory Effect of Aldosterone and Cortisol on the Work of the Isolated Rat Heart-Lung.
Kathyrn W. Ballard (by invitation) and *George Sayers.*

72. The Hemodynamic Parameter Mediating Increase in Aldosterone Secretion.
Donald S. Gann (by invitation), *Ivor H. Mills* (by invitation) and *Frederic C. Bartter.*

73. Preliminary Characterization of Glomerulotropin.
Gordon Farrell, Avery D. Pratt, Jr. (by invitation), *Laurence S. Harris* (by invitation) and *Robert C. Banks* (by invitation).

74. Changes in Metabolic Activities of Steroid Hormones Associated with Methylation and Hydroxylation in Ring D.
Maurice M. Pechet, Evelyn L. Carroll (by invitation) and *Anne P. Forbes.*

75. Effect of Walker Carcinoma on Steroid Diabetes in Rat.
David J. Ingle (introduced by Dwight J. Ingle).

76. Acute Effects of Adrenalectomy and Hydrocortisone on Glucose Tolerance of Diabetic and Eviscerate Rats.
O. K. Smith and *C. N. H. Long.*

INTERMISSION

77. Influence of Age, Adrenalectomy and Cortisol Administration on the Activity of Hepatic Glutamic-Pyruvic Transaminase.
Homer Harding, Fred Rosen and *Charles Nichol* (introduced by Avery A. Sandberg).

78. The Metabolism of 2 C^{14} Pyruvate in Normal and Adrenalectomized Rats.
William W. Winternitz.

79. Observations on the Role of the Adrenal Gland in the Metabolic Response to Surgery.
Maureen Smith, H. A. F. Dudley, J. S. Robson and *C. P. Stewart* (introduced by Grant W. Liddle).

80. Renal-Cardiovascular Pathology in Aging Rats With and Without Exposure to ACTH and Stressors.
George F. Wilgram (by invitation) and *Dwight J. Ingle.*

81. The Effects of Anoxis on Adrenal Cortical and Medullary Secretion in the Dog.
Alan Goldfien and *William F. Ganong.*

82. Effect of Adrenalectomy and Cortisone on Rate of Eruption of Maxillary Incisors in Female Rats.
L. V. Domm, W. A. Wellband (by invitation) and *J. P. Howard* (by invitation).

IX. 2:00 P.M.—INSULIN AND DIABETES

Carolina Room, Chalfonte

Chairmen: *S. Fajans and A. Renold*

83. Differential Resin Binding of Insulin in Sera from Insulin Responsive and Resistant Diabetic Subjects.
Marvin L. Mitchell and *Mary E. O'Rourke* (by invitation).

84. Serum Insulin-Like Activity in Patients with Pancreatic Islet-Cell Tumors.
Yves M. Dagenais (by invitation), *Albert E. Renold, Donald B. Martin* (by invitation) and *Vilma Lauris* (by invitation).

85. Role of Individual Organs in Carbohydrate Metabolism in Intact Normal Dogs.
Dorothy H. Henneman, William Shoemaker (by invitation) and *William Walker* (by invitation).
86. Hepatic Glucose Metabolism in Two Forms of Spontaneous Hyperglycemia.
Gaston R. Zahnd, Jean Christophe and *George Yerganian* (introduced by Albert E. Renold).
87. Carbohydrate Metabolism in Amphibians and Reptiles.
Malcolm R. Miller (Introduced by Frank L. Engel). (Invited paper, 20 minutes.)

INTERMISSION

88. On the Mechanism of the Blood Glucose Lowering Action of Insulin.
N. Altszular, A. Dunn (by invitation), *R. Steele, D. T. Armstrong* (by invitation), *J. S. Bishop* (by invitation) and *R. C. de Bodo.*
89. An Insulin Effect on Peptide Synthesis Independent of Glucose or Amino Acid Transport.
Ira G. Wool and *M. E. Krahl* (by invitation).
90. Comparison of Epinephrine and Insulin on Adipose Tissue Metabolism.
George F. Cahill, Jr., Bernard Leboeuf (by invitation), and *Robert Flinn* (by invitation).
91. The Action of Insulin, Tolbutamide and Hypoglycin A on the Adipose Tissue of Rats.
Thomas C. Smith, Lena Will (by invitation), and *Ira Ringler.*
92. Relation of Insulinogenesis to Pancreatic B Cell Acid and Glucose-6-Phosphatase.
Sydney S. Lazarus (introduced by Bruno W. Volk).
93. Effect of Glucagon on Hepatic Blood Flow and Metabolism in Unanesthetized Dog.
W. C. Shoemaker and *T. B. Van Itallia* (introduced by Jay J. Gold).

TO BE READ BY TITLE

See Abstracts 94–170

GORDON RESEARCH CONFERENCE

CHEMISTRY, PHYSIOLOGY AND STRUCTURE OF BONES AND TEETH

13–17 July 1959
Kimball Union Academy
Meriden, New Hampshire

W. D. Armstrong, Chairman
B. B. Migicovsky, Vice Chairman

13 July

Invited Short Communications
Felix Bronner, Chairman
Bone as a Mechanical Tissue
Jonathan Cohen, Chairman
Bone as a Problem in Mechanical Engineering

14 July

F. G. Evans. The Relation of the Microscopic Structure of Bone to Its Strength Characteristics and Other Physical Properties

H. Hollingshaus. Mechanical Properties of Bone From Radium Poisoned Dogs
W. T. Dempster. The Distribution of Osseous Material in the Mandible and Humerus, and Its Mechanical Importance
M. G. Hardinge. The Transmission of Weight Through the Knee Joint

Fluoride Metabolism
Harold C. Hodge, Chairman
D. A. Greenwood. Broad Aspects of Fluoride Metabolism
Yngve Ericsson. Radiofluoride Studies

15 July
P. H. Phillips. Bone Retention of Fluoride
C. S. Hobbs. Radiofluoride Distribution in Animals Under Chronic Fluoride Exposure
E. J. Largent. Urinary Fluoride Levels in Relation to Change in Osseous Radiopacity
F. J. McClure. Fluoride Content and the Chemistry of Human Skeletal Tissue
C. H. Carlson. Mechanisms of Renal Excretion of Fluoride Determined with Radiofluoride
Leon Singer. Plasma Fluoride Concentrations in Relation to Fluoride Intake

New Developments in Methodology in Study of Calcified Tissues
D. Dziewiatkowski, Chairman
Diego Carlstrom. Some New Aspects of Ultrastructure of Bone

16 July
James Irving. Histochemical Changes at Sites of Calcification
Richard Greulich. High Resolution Microradiography
Harald Schraer. Quantitative Determination of Bone Mineral Content and Bone Density In Vivo From Standardized Roentgenograms

Pathological Physiology of Bone and Calcium Metabolism
Franklin C. McLean, Chairman
G. Fanconi. Idiopathic Hypercalcemia

17 July
Albert Dorfman. Studies on Acid Mucopolysaccharides
Donald Fraser. Hypophosphatasia
Karl M. Wilbur. Mechanisms of Mineralization in Molluscs

Attendance. Requests for attendance at the conferences, or for additional information, should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From 15 June to 4 September 1959 mail should be addressed to Colby Junior College, New London, New Hampshire.

Registration and Reservations. Attendance at the Conferences is by application. Individuals interested in attending the conferences are requested to send their applications to the Director at least two months prior to the date of the Conference. *All applications must be submitted on the standard application form which may be obtained by writing to the office of the Director.* This procedure is important because certain specific information is required in order that a fair and equitable decision on the application may be made. Attendance at each Conference is limited to approximately 100 individuals.

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STIMULATION OF RAT ADRENOCORTICAL SECRETION BY A FACTOR PRESENT IN HUMAN URINE

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ABSTRACT

Production of corticosteroids by the rat adrenal gland has been studied in vitro. Both a commercial and a highly purified preparation of ACTH stimulated the secretion of "corticosterone" and aldosterone *in vitro*. The "corticosterone" fraction was increased more than aldosterone. Addition of the non-ultrafilterable fraction of human urine to the incubate stimulated the secretion of aldosterone more than "corticosterone." Several possible interpretations of this effect are discussed.

THE rat adrenal gland has been demonstrated to produce corticosterone and aldosterone *in vitro* (1, 2). Stimulation of this production by ACTH has also been shown (1, 2). The technique, therefore, offers an opportunity to study factors directly influencing either glucocorticoid or mineralocorticoid secretion. In the present study, the non-ultrafilterable fraction of human urine has been assayed for its effect on adrenocortical secretion.

METHODS

Adrenal glands from 60 to 80 rats were trimmed of fat, bisected, weighed, and apportioned equally to the control and experimental flasks. Each flask received one-half of an adrenal from each rat. For 25 mg. of tissue, 1 cc. of Krebs-Ringer bicarbonate buffer with glucose, 200 mg. per 100 cc., was added to the flasks. The flasks were incubated in a Dubnoff metabolic incubator at 37.5° C in an atmosphere of 95% O_2 and 5% CO_2. After one-half hour of incubation, the medium was poured off and discarded. An equal quantity of fresh medium, containing the material to be tested, was added and incubation was continued for two hours. Each medium was extracted with several volumes of redistilled chloroform. The chloroform extract was chilled, washed once with cold 0.05N NaOH, and twice with cold distilled water, dried with anhydrous sodium sulfate and evaporated *in vacuo* at 40° C. The residue was applied to paper and chromatographed

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in the chloroform/formamide system, using reference standards of cortisol, cortisone, and corticosterone. A contact photoprint of the chromatogram was made using a G.E. Sterilamp as a light source. The fractions indicated by the major ultraviolet-absorbing bands and by the reference standards were eluted with ethanol. Formamide was removed by partitioning the residue between water and chloroform. In some instances, fractions were rechromatographed in other systems. The ultraviolet absorption peak near 240 mμ was used to detect and measure alpha, beta-unsaturated ketones. Soda fluorescence (3), reduction of blue tetrazolium (3), and Zimmerman reaction (4) were measured in several fractions. Aldosterone was estimated by bioassay in adrenalectomized rats (5), using a ratio of activity of aldosterone to desoxycorticosterone acetate of 36.5 to 1.

Human urine, preserved by refrigeration, was ultrafiltered through a 2% collodion membrane (6). The protein was eluted from the membrane by the method of Rigas and Heller (7), using isotonic saline. Urine "protein" A and B were lyophilized pools of the urine protein obtained from 100 and 75 patients, respectively. Urine "protein" C and E were each a 48-hour pool from two individuals. Urine "protein" F was a pool from 20 normal adults.

Urine "protein" A_D was a quantity of urine "protein" A which was dialyzed in the cold against distilled water.

RESULTS

Some characteristics of the fractions consistently separated by paper partition chromatography are presented in Table 1. Although not conclusively identified, corticosterone and aldosterone are presumed to be present.

TABLE 1. CHROMATOGRAPHIC BEHAVIOR AND SPOT TESTS OF ADRENAL SECRETION

System: Chloroform-foramide	Rechromatographed in:
Fraction I *Rf:* One narrow band: U-V absorption (234 mμ) ——→ Slightly more polar than cortisol	*Bush C* One band, distinctly more polar than cortisol. Has soda fluorescence. Reduces blue tetrazolium slowly. Negative Zimmermann reaction
Fraction II *Rf:* Narrow, faint band of U-V absorption Runs near cortisone. Reduces blue tetrazolium Strong mineralcorticoid on bioassay presumed to be aldosterone	
Fraction III-IV *Rf:* Broad band near corticosterone "B fraction" ——→	*Toluene-propylene glycol* Two bands of U-V absorption (240 mμ) *Fraction III* = More polar than corticosterone Does not reduce blue tetrazolium; Has soda fluorescence. Negative Zimmermann reaction *Fraction IV* = Presumed to be corticosterone. Reduces blue tetrazolium; has soda fluorescence
Fraction V *Rf:* Broad band near solvent front	

TABLE 2. ACTH

Experiment	*Dose/Flask*	μg. aldosterone eqv./100 mg. tissue/2 hrs.	%Δ	μg. B eqv. /100 mg. tissue/2 hrs.	%Δ
Control		0.15	+135	8.3	+422
ACTH (Armour)	4.0 units	0.36		43.3	
Control		0.20	+ 65	3.9*	+254
ACTH (Armour)	3.8 units	0.33		13.8*	
Control		0.25	− 7	6.7	+ 75
ACTH† (C. H. Li)	40 μg.	0.23		11.7	
Control		0.13	+112	6.6	+435
ACTH† (C. H. Li)	200 μg.	0.28		35.4	

Mean diff. test-control 0.12∓0.04 (S.E. of mean). Significant at $P < .05$ (t test).
* Only Corticosterone measured.
† Sheep ACTH.

EFFECT OF ADRENOCORTICOTROPHIN (ACTH)

Table 2 contains data from two experiments with lyophilized bovine ACTH (Armour) and two with a highly purified sheep ACTH prepared by C. H. Li and co-workers (8). The "B fraction" represents the sum of the two components into which the band moving with corticosterone in the chloroform/formamide system separates when rechromatographed in toluene/propylene glycol. Both of these components appeared in increased amount when ACTH was added. Although secretion of aldosterone and the "B fraction" is usually increased by ACTH, the increase in "B fraction" is greater both absolutely and relatively.

EFFECT OF NON-ULTRAFILTRABLE FACTOR OF HUMAN URINE

Results of experiments in which urine "protein" was added to the media are presented in Table 3. Secretion of aldosterone is regularly increased by addition of the urine "protein." Although the degree of effect varies markedly among experiments, the effect is consistently in the same direction and highly significant ($P < .005$). Moreover, the pattern of corticosteroid secretion is different from that noted with ACTH. Aldosterone secretion is consistently stimulated to a greater degree than the "B fraction."

The variation in the values of the control adrenals among experiments is large. No attempt was made to control weight, sex of rats, or seasonal factors from experiment to experiment. This may well account for the variation. However, in an individual experiment, the adrenals of the control and experimental flasks were relatively homogeneous in that each flask contained one half of an adrenal from every rat and the same number of left and right adrenal halves.

Four different pools of urine "protein" showed some activity. In one experiment a dose-response effect could not be established. Possibly, the lowest dose of urine protein used produced a maximal stimulation. There

TABLE 3. HUMAN URINE PROTEIN

Experiment	Dose/Flask	μg. aldosterone eqv./100 mg. tissue/2 hrs.	%Δ	μg. B. eqv./100 μg. tissue/2 hrs.	%Δ
Control		0.08			
Urine Protein A	20 hrs.	0.22	+166	6.0	
Control		0.33		3.6	
Urine Protein A	20 hrs.	0.43	+ 30	4.2	+17
Control		0.085		5.7	
Urine Protein A	5 hrs.	0.26	+205	7.0	+22
Urine Protein C	12 hrs.	0.31	+261	7.7	+35
Control		0.18		7.2	
Urine Protein A	17 hrs.	0.47	+263	7.8	+08
Urine Protein A_D	17 hrs.	0.65	+365	8.0	+11
Control		0.72		7.4	
Urine Protein A	5 hrs.	1.20	+ 66	6.6	−11
Urine Protein A	10 hrs.	0.86	+ 19	7.6	+ 3
Urine Protein A	20 hrs.	0.86	+ 19	7.8	+ 5
Control		0.16		3.9	
Urine Protein B	5 hrs.	0.23	+ 43	4.7	+20
Urine Protein E	10 hrs.	0.26	+ 64	4.9	+25

Test-control mean diff. $=0.19 \pm 0.045$ (S.E. of mean). $P < .005$ by t test.

was no obvious explanation for the very high control level of aldosterone secretion in this experiment. The urine "protein" which had been dialyzed against distilled water showed activity greater than before dialysis.

CONTROL EXPERIMENTS

To study the specificity of this effect, several different experiments were carried out. These are recorded in Table 4. In the first experiment, a 20-hour dose of urine "protein" A was added to a flask containing incubation media without adrenals. The flask was incubated and processed in the usual manner. No aldosterone was detected by bioassay, nor other corticosteroids by ultraviolet absorption, blue tetrazolium reaction and soda fluorescence. This eliminated the possibility that aldosterone bound to the urine protein was being added to the media.

In the second experiment, a quantity of urine "protein" subjected to prolonged boiling was added to a flask with rat adrenals. An equal quantity of untreated urine "protein" was added to another flask. Three pools of urine "protein" were studied in this fashion. In each instance, the untreated urine "protein" promoted a greater output of aldosterone than the boiled urine "protein." In the two experiments with urine "protein" A and urine "protein" F, this increment was outside the 95% confidence limits of the bioassay.

Another experiment shows the results of addition of purified beef growth hormone and highly purified pig melanocyte-stimulating hormone to the incubation media of rat adrenal glands. In the dose used, it was not shown

TABLE 4. CONTROL EXPERIMENTS

Experiment	Dose/Flask	μg. aldosterone /100 mg. tissue /2 hrs.	%Δ	μg. B. fraction /100 mg. tissue /2 hrs.	%Δ
Urine Protein A	20 hrs. $\bar{S}$ adrenals	0		0	
Boiled Urine Protein A	20 hrs.	0.204		4.5	
Urine Protein A	20 hrs.	0.296*	+45	3.7	−18
Control		0.157		3.9	
Boiled Urine Protein B	5 hrs.	0.190	+21	5.3	+36
Urine Protein B	5 hrs.	0.225	+43	4.7	+20
Control		0.131		—	
Boiled Urine Protein F	17 hrs.	0.150	+14	—	
Urine Protein F	17 hrs.	0.386	+197	—	
Control		0.250		6.7	
Growth Hormone	400 μg.	0.207	−17	8.3	+24
MSH	700 μg.	0.313	+25	9.2	+49
Control		0.094		8.1	
Albumin	40 mg.	0	—	6.6	−18
Gelatin	40 mg.	0	—	4.4	−56

* Assay result was above upper limit of dose response curve. Therefore, at least this quantity of aldosterone was present.

that either hormone affected the secretion of aldosterone significantly.

Gelatin, U.S.P., and four-year old human serum albumin, prepared by Cutter Laboratories, did not stimulate aldosterone production and appeared to depress "corticosterone" secretion.

DISCUSSION

In the present studies, the yield of aldosterone was much less than that reported by Giroud *et al.* (2). The yield of corticosterone, which comprises approximately half of the B fraction, was closer to the quantity he reported. Strain, sex of rat, seasonal variations, or technique may explain this discrepancy.

Addition of the crude protein fraction from human urine to the incubation media of rat adrenal glands *in vitro* promotes an increase in aldosterone secretion. This increment is greater than that of the "corticosterone" fraction. Addition of ACTH causes a different pattern of secretion, namely, a marked rise in the "corticosterone" fraction with a moderate increase in aldosterone.

Four possible explanations of this effect are: (a) It is a non-specific effect. (b) Necessary nutrients, missing from the artificial media, are supplied with the biological fluids. (c) A minute quantity of ACTH present in the biological fluids is producing the observed results. (d) A factor present in human urine specifically stimulates the secretion of aldosterone.

Experimental results presented in Table 4 are evidence against a non-specific effect. Also, addition of the urine protein or serum does not change the potassium to sodium ratio of the media, nor does dialysis destroy the activity of the urine protein. However, failure to establish a dose-response curve is compatible with a non-specific effect.

Presence of nutrients in the biological fluids which are necessary for proper metabolism of adrenal glands *in vitro* cannot be ruled out. Extensive studies by Schonbaum *et al.* (9) found no substance that significantly increased the basal secretion or the response to ACTH of rat adrenals when added to the basic medium (Krebs-Ringer bicarbonate buffer with added glucose). Nevertheless, characterization of such nutrients, if they exist, still would be of biological significance.

In a single *in vitro* experiment by Giroud, *et al.* (1) doses of 1 and 100 milliunits of commercial ACTH preparation stimulated the secretion of aldosterone to the same extent. The 1 milliunit dose has no effect upon secretion of total Δ^4, 3-ketosteroids. Measurement of total Δ^4, 3-ketosteroids, however, includes a number of compounds which are increased little, if at all, by ACTH. Therefore, a small increment in corticosterone secretion might not be detectable. In one experiment by us, a quantity of a highly purified ACTH preparation sufficient to cause a 75% increase in secretion of the "B" fraction had no effect on aldosterone.

If the observed increase in aldosterone secretion is a specific effect of a humoral factor, the possibility arises that this may play a physiological role. Two other lines of evidence lend support to the hypothesis of a humoral factor controlling secretion of aldosterone. Injection of a beef diencephalic extract into dogs has been shown by Farrell *et al.* (10) to stimulate preferentially secretion of aldosterone. Whole urine from sodium-depleted adrenalectomized rats was found by Ralli *et al.* (11) to cause sodium retention in rats with adrenals, but not in adrenalectomized rats.

The present study indicates a humoral factor may influence secretion of aldosterone. Further experiments are in progress to delineate the nature and physiological role of this factor.

Acknowledgments

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ADRENOCORTICAL INFLUENCES UPON THE RECONSTITUTION OF LYMPHATIC TISSUE FOLLOWING ACUTE INVOLUTION[1]

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ABSTRACT

A single subcutaneous injection of an intoxicating dose of ethyl alcohol, given to intact 17 week old male CBA mice, induced an acute involution of the thymus which was maximal 36 to 48 hours after the injection. Based on a comparison made with the thymo-regressive response to hydrocortisone, the involution observed was estimated to have been due to a fifty-fold increase in adrenocortical steroid activity. A comparison of the growth curves and the cytological mechanisms responsible for the reconstitution of the thymus was then made, between the stressed-intact animals and stressed animals bilaterally adrenalectomized at the time of maximal thymic involution. In the intact animals, the thymus regained 50% of its total weight loss by the 96th hour after the alcohol injections. Beyond this the rate of reconstitution decreased and the normal thymic weight was not attained until between the 240th and 288th hours. Adrenalectomy completely reversed the shape of the growth curve. There was an initial inhibition of growth and a delayed but positively accelerated growth resulting in a hyperplasia of the thymus on the 288th hour. The differences in the growth curves were associated with differences in the cytological mechanisms responsible for growth. It is suggested that there may exist adrenocortical influences which exert stimulating, as well as moderating, effects, upon the reconstitution of the thymus following acute involution.

RECENT investigations concerning the role of the pituitary-adrenal system in acute alcoholism have demonstrated that acute alcohol intoxication induces a depletion of the adrenal ascorbic acid and cholesterol content in intact rats (1, 2) and guinea pigs (3, 4, 5). An acute intoxication induced by the intraperitoneal injection of ethyl alcohol, was also found to induce an involution of the thymus in intact but not in adrenalectomized mice. This phenomenon was then utilized to arrive at a quantitative estimate of the increase in adrenocortical activity associated with the alcohol intoxication. This was done by comparing the thymic involution obtained in intact mice, given two intraperitoneal injections of alcohol, with that obtained in adrenalectomized animals treated with graded doses of hydrocortisone (6).

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The present report deals with studies designed to determine the influences of the adrenal cortical secretions upon the reconstitution of the thymus following the acute involution induced by a single subcutaneous injection of an intoxicating dose of ethyl alcohol. These studies involve the determination of the time required for maximal thymic involution to occur; the estimation of the increase in adrenocortical activity associated therewith; and the study of the reconstitution of the normal mass and cytology of the thymus following the acute involution in intact alcohol-stressed animals. The adrenocortical influences upon the reconstitution of the thymus following acute involution were then determined by comparing the growth curve of the thymus of the alcohol-stressed intact animals with that of alcohol-stressed animals bilaterally adrenalectomized at the time of maximal thymic involution (36 hours post-stress).

EXPERIMENTAL

A total of over 700 male CBA mice 17 weeks of age ±4 days were used in these studies. These animals were bred in our own colony and were kept under optimal laboratory conditions at all times. Over 30 experiments were performed during a period of 15 months using 20 to 30 animals in each experiment.

The degree and duration of acute involution following alcohol treatment and the time required for the reconstitution of the thymus were first determined. The animals used in this phase of the study are designated as "stressed-intact." As large enough groups of animals came of age, they were treated with a standard intoxicating alcohol dose (6000 mg./kg. of body weight) and groups of at least four animals were sacrificed at various intervals after the injections. At the time of killing, the wet weights of the thymi, peripheral lymph nodes (axillary and inguinal) and paired adrenal glands were determined. These were dissected free of fat and connective tissue and weighed on a Roller-Smith torsion balance accurate to the nearest 0.1 mg. These procedures were repeated until a complete curve of acute involution and reconstitution of the thymus was obtained with at least seven samples from two separate experiments at each time interval following the injection. Initial and final body weights and the losses in body weights were determined for all animals.

The alcohol used was a 50% dilution of absolute ethyl alcohol, U.S.P. and 0.85% pyrogen-free saline. The standard dose of alcohol was volumetrically adjusted, according to the body weight of each animal. This dose, injected subcutaneously, induced an acute intoxication with a resultant mortality of approximately 50% within the first 24 hours. This dose was found to be twice the dosage required to obtain similar intoxicating and lethal effects using intraperitoneal injections as reported in a previous publication (6). The subcutaneous route of administration was used in these studies because it was found that intraperitoneal injections led to a 100% mortality before the thymus returned to normal.

To determine the influence of adrenalectomy upon thymic reconstitution, groups of animals were bilaterally adrenalectomized at the time of maximal thymic involution (36 hours following the alcohol injection); and the subsequent growth curves of lymphatic organs were determined using the standard techniques for the determination of lymphatic organ weights. This group is referred to as "stressed-adrenalectomized."

An estimate of the increase in adrenocortical activity associated with the alcohol-induced stress was obtained by determining the dose of hydrocortisone (Merck) required to induce a thymic involution in adrenalectomized animals equivalent to the maximal involution observed in stressed-intact animals. This was done using the same techniques as reported in a previous study (6).

Organ weights of all experimental animals were expressed in absolute and relative (mg./100 gm. of initial body weight) terms. The control body and organ weights are the means obtained from intact non-stressed animals sacrificed at the start and termination of each separate experiment. Lymphatic organs were fixed in Zenker-formol and stained routinely with hematoxylin and eosin, and methyl-green pyronin for histological study.

RESULTS

A. *Stressed-intact mice*

Body weight. Following the alcohol injections there was a depression in body weights. This became maximal at 36 and 48 hours post-stress, beyond which the difference between initial and final body weights decreased (Table 1). Beyond the 168th hour post-stress, normal body weight was virtually restored.

Thymus. From a mean control weight of 27.6 ± 1.5 mg. (absolute) and 106.2 ± 5.8 mg. (relative), the thymus lost approximately 63% absolute and relative weight by the 36th hour after injection. Beyond the 48th hour interval there occurred a rapid growth of this organ so that by the 96th hour the thymus regained approximately 50% of the weight loss observed at 48 hours (Fig. 1). Beyond the 96th hour the growth rate gradually decreased and the normal thymic weight was not restored until between the

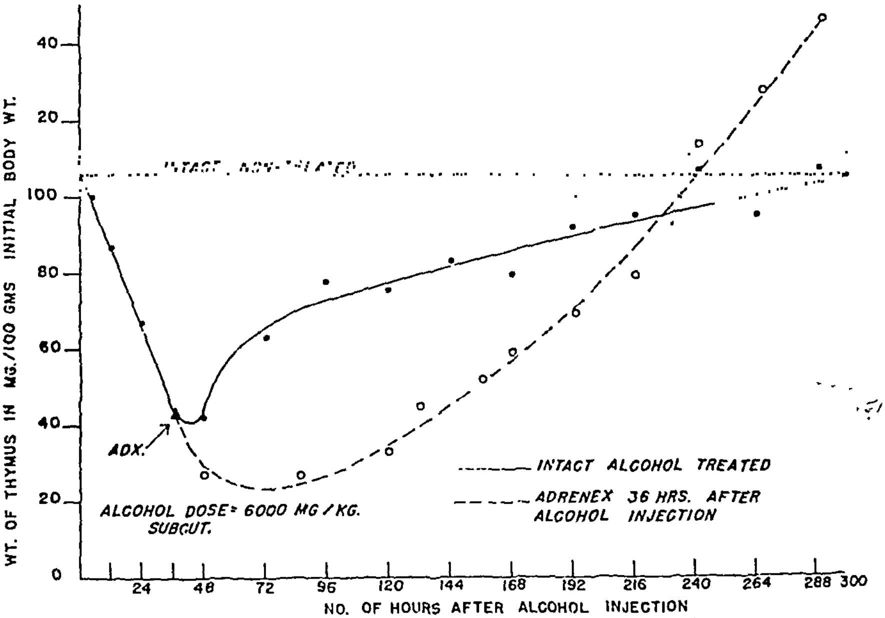

FIG. 1. Curves of involution and reconstitution of the thymus of alcohol-injected *CBA* mice, intact and adrenalectomized 36 hours after alcohol injection.

TABLE 1. BODY AND ORGAN WEIGHTS OF INTACT MICE AT VARIOUS INTERVALS AFTER A SINGLE ALCOHOL INJECTION, AND FOLLOWING ADRENALECTOMY PERFORMED AT THE THIRTY-SIXTH HOUR AFTER ALCOHOL TREATMENT*

	Hrs. post inj.	Hrs. post adx.	Body weights, gm.†			Organ weights, mg.†					
			Initial	Final	Loss	Thymus		Lymph nodes		Adrenals	
						Absol.	Rel.‡	Absol.	Rel.	Absol.	Rel.
Alcohol injected—Intact	0		24.9± .3			27.6±1.5	106.2± 5.8	10.1± .3	40.4±2.0	2.8±.1	
	4		25.1± .8	24.8± .8	.3± .2	25.2±1.9	99.9± 1.8	9.9± .5	40.2±2.4	3.1±.2	12.5± .7
	12		23.0± .4	22.2± .6	.8± .2	20.0± .9	86.8± 3.1	7.8± .8	34.4±4.4	3.0±.2	12.8± .8
	24		24.9± .8	23.0± .7	1.9± .2	16.9±1.2	67.2± 3.8	7.8± .7	30.4±2.2	2.6±.2	10.7± .8
	36		24.6± .3	22.0± .3	2.6± .2	10.3± .8	43.2± 3.4	9.5± .8	37.5±3.6	3.3±.3	13.9±1.3
	48		25.1±1.0	22.9±1.0	2.2± .2	10.5±1.2	42.2± 4.9	7.8± .8	32.5±3.1	3.6±.4	13.7±1.1
	72		24.9± .6	24.2± .4	1.4± .2	16.8±1.2	63.5± 5.2	9.8± .5	37.7±2.4	2.9±.2	11.0± .6
	96		24.2±1.0	23.5± .8	.7± .2	18.9±1.6	78.3± 6.5	9.2± .7	38.0±2.0	2.6±.2	10.8± .5
	120		24.4± .8	23.7± .7	.7± .1	18.5±1.1	75.8± 4.6	10.6± .5	43.3±1.7	2.9±.1	12.1± .9
	144		25.0± .9	23.9± .9	1.1± .3	20.5±1.0	82.7± 4.0	10.5± .5	41.2±1.9	2.8±.2	10.7± .6
	168		25.3± .5	24.9± .2	.4± .2	20.0±1.3	79.3± 5.3	10.7± .7	43.6±2.5	3.2±.1	12.5± .6
	192		26.0± .6	24.8± .5	1.2± .1	24.0±1.2	92.0± 3.6	17.6±1.3	66.0±3.9	2.8±.1	10.8± .5
	216		26.0± .5	25.1± .6	.9± .2	24.7±1.3	95.0± 4.5	16.0± .6	54.9±2.3	3.0±.3	11.6±1.2
	240		27.4±1.1	26.5± .7	.9± .2	29.5±2.2	106.0± 5.5	16.0±1.5	59.8±8.3	3.1±.2	11.2± .5
	264		28.4± .9	27.9± .9	.5± .2	28.6±2.4	95.0± 4.5	14.5± .3	49.2± .9	2.8±.1	9.7± .3
	288		26.5± .9	26.0± .8	.5± .2	28.2±1.7	106.0± 4.1	19.7±1.5	79.4±5.8	2.6±.3	10.1±1.0
Alcohol injected—Adrenalectomized	36	0	24.6± .3	22.0± .3	2.6± .4	10.3± .8	29.5± 1.5	9.5± .8	37.5±3.6		
	48	12	25.0± .7	22.2± .6	2.8± .4	6.2± .7	27.1± 2.7	4.8± .6	18.8±2.3		
	84	48	28.0± .5	23.8± .5	4.2± .6	7.6± .6	27.7± 2.5	10.8±1.4	39.1±6.3		
	120	84	24.6± .9	21.1± .9	3.5± .4	8.5± .5	33.3± 2.9	7.6± .3	31.7±1.8		
	132	96	24.1±1.2	20.4±1.0	3.7± .6	11.1±2.2	45.1± 7.7	10.4± .6	42.3±5.0		
	156	120	25.5± .2	21.9±2.5	3.6±1.2	11.8±1.1	52.3± 3.2	10.5± .7	41.3±4.2		
	168	132	26.1±1.0	19.6± .3	6.5± .7	10.2±1.2	59.4± 4.9	10.4± .9	38.5±3.7		
	192	156	24.7± .6	21.5± .9	3.2±1.2	17.4±3.8	69.9± 4.4	9.1± .9	36.4±3.6		
	216	180	24.4± .9	20.8± .9	3.6± .6	22.8±3.5	94.5±13.9	11.9±1.9	49.6±8.9		
	240	204	24.2± .5	23.1±1.0	1.1± .9	27.0±4.4	111.9± 9.5	10.1±3.9	34.3±9.9		
	264	228	26.1± .3	22.9± .4	3.2±1.0	33.6±5.3	127.4± 9.9	11.7±1.6	43.9±6.6		
	228	252	27.3± .4	22.8± .7	4.5± .5	37.0±2.0	147.4± 9.8	16.2±1.9	60.6±8.7		

* 6000 mg./kg. body weight, subcutaneously injected.
† Mean and S.E., seven animals/group.
‡ Mg./100 gm. initial body weight.

240th and 288th hour. Histologically it was found that during the initial rapid growth phase (between 48 and 96 hours) there occurred a proliferation of the reticular cells in the thymic cortex (Fig. 2), whereas during the slower growth phase (beyond the 96th hour) there occurred a differentiation of the reticular cells and proliferation of the lymphoid elements, leading toward the reconstitution of the normal cytology of the thymic cortex consisting mostly of small and medium-sized lymphocytes.

Peripheral lymph nodes and adrenal gland. No significant changes were observed in the weights of the peripheral lymph nodes during the first 168 hours following the alcohol stress; however, beyond this point the peripheral lymph nodes underwent a gradual increase in weight so that a significant hyperplasia of these organs was manifest at the 288th hour (Table 1). The adrenal gland failed to undergo significant weight alterations throughout the entire period of observation.

B. *Stressed-adrenalectomized mice*

Body weight. Examination of the changes in body weight in the stressed adrenalectomized animals (Table 1) shows that there occurred an additional loss of body weight by the 48th hour after the operation, at which time there was a loss of 4.2 ±.6 gm. Beyond the 48th hour after adrenalectomy the body weight remained 3.5 to 6.5 gm. below normal.

Thymus. Within the first 12 hours following adrenalectomy there occurred an additional loss in the absolute and relative thymic weights (Table 1). The thymus remained small until the 96th hour (132nd hour post-stress). Beyond this hour, there occurred a rapid growth which continued until the thymus of the stressed-adrenalectomized animals was greater than that of the non-stressed controls on the 264th and 288th hour after the injection (Fig. 1). Due to the high mortality we have been unable to pursue our study beyond the 288th hour.

Histological studies revealed that during the latent period (up to the 96th hour post-adrenalectomy) preceding the phase of rapid growth which occurred between the 96th and 252nd hour, there was no cytological evidence of growth. During this period there were a few reticular cells and a greater relative number of lymphoid elements in the thymic cortex (Fig. 3). During the later phase of rapid growth there occurred a proliferation of the residual lymphoid elements but at no time was there observed a preponderant increase in the reticular cells as was observed during the initial period of rapid growth in the stressed-intact animals.

Lymph nodes. Following adrenalectomy there occurred an additional loss in the weight of the peripheral lymph nodes so that within 12 hours after adrenalectomy (48 hours post-stress) the lymph nodes were significantly smaller. However, by the 48th hour after the operation the lymph nodes returned to their normal absolute and relative weights and remained at normal level for the next 179 hours. A significant increase in lymph node weights was observed at the 288th hour.

C. *Increase in corticoid activity*

The combined results of two experiments designed to obtain an indirect estimate of the increase in adrenocortical activity as induced under the conditions of this study are presented in Figure 4. An estimate of corticoid secretion was made by determining the dose of hydrocortisone required to induce a thymic involution in adrenalectomized animals equivalent to that obtained in intact animals 36 hours after the alcohol injection. In Figure 4, the relative weights of the thymus are plotted against the log-dose of administered hormone. The horizontal shaded bars represent the mean and standard error of the mean thymic weights of the intact non-treated and of the intact alcohol-treated animals, as obtained 36 hours after the start of the experiment. The sloped line is the thymo-regressive response line mathematically fitted about the observed relative weights obtained from groups of animals which were adrenalectomized, 24 hours later treated with a single injection of one of a graded series of doses of hydrocortisone,

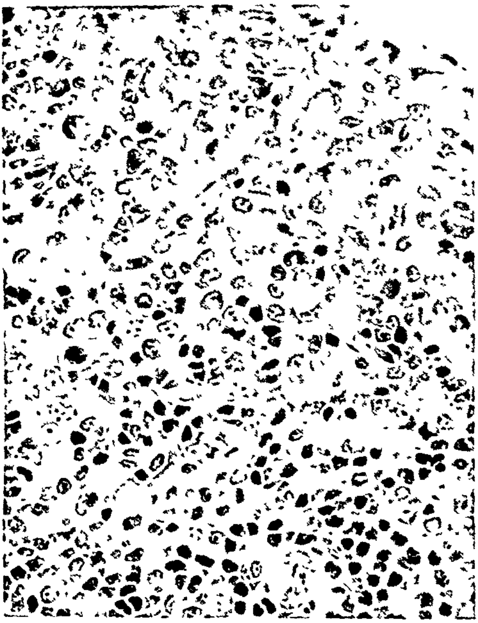

FIG. 2. Thymic cortex of an intact alcohol-treated mouse 72 hours after alcohol injection. Note the large number of normal immature lymphocytes and reticular cells as compared to Figure 3. H. and E. ×500.

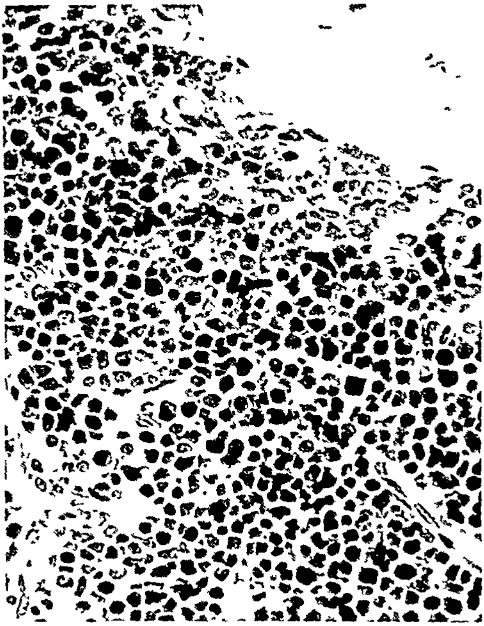

FIG. 3. Thymic cortex of alcohol-treated adrenalectomized mouse, 72 hours after alcohol injection, 36 hours after adrenalectomy. Note the large numbers of small and medium hyperchromatic lymphocytes and few reticular cells as compared to Figure 2. H. and E. ×500.

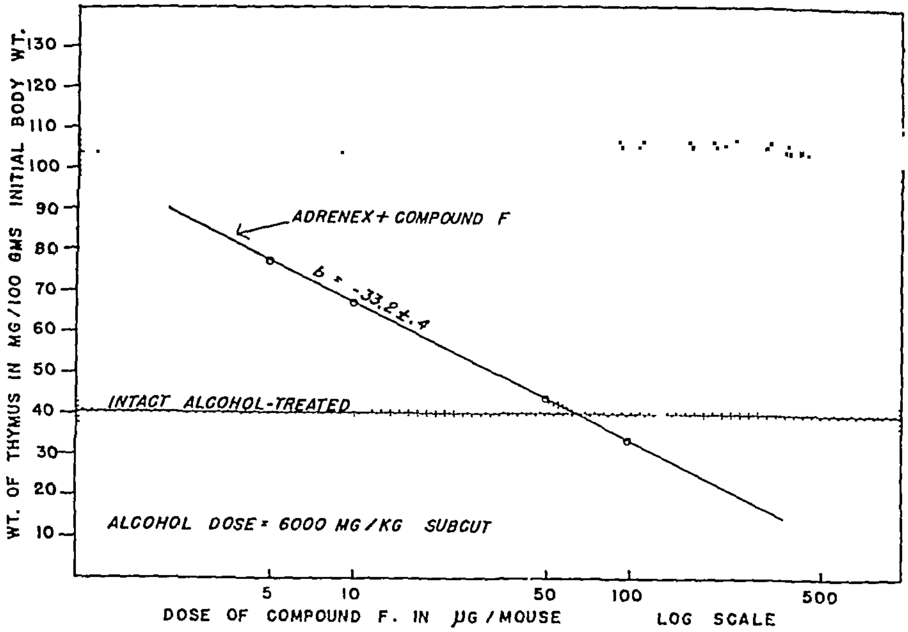

FIG. 4. Log dose-response relationships between the relative weight of the thymus and graded doses of hydrocortisone in adrenalectomized CBA mice. Shaded bands represent mean and S.E. of the thymic weights of intact non-treated and intact alcohol-treated groups.

and sacrificed 36 hours after the hormone treatment. From the intersection of the thymo-regressive response line with the line representing the thymic weights of the control and alcohol-treated animals, it may be seen that a dose of over 50 micrograms of compound F induced a thymic involution equivalent to that induced by the alcohol treatment during an equivalent period of time. This represents about a fifty-fold increase in the hormone dose required to maintain the thymic weight at normal levels in adrenalectomized mice. From this relationship it can be assumed that the subcutaneous alcohol injections in intact animals induced a fifty-fold increase in adrenocortical activity. This agrees with the estimate of increase in corticoid activity arrived at using intraperitoneal injections of alcohol in female mice 10 weeks of age as reported in a previous communication (6).

DISCUSSION

There is an extensive amount of literature concerning the hormonal control of lymphatic tissue (7). For the most part the available information is concerned with those hormones or conditions which favor acute involution, but there is little information concerning the factors which stimulate the growth of lymphatic tissue, either from normal levels or following acute involution. The results presented in this report strongly suggest that there exist adrenal cortical influences which stimulate or enhance the reconstitution of the thymus following acute involution. This is evidenced by the inhibition and alteration in growth of the thymus which occurred following adrenalectomy performed at the time of maximal stress-induced acute involution.

The mechanisms by which the adrenal cortex may influence the growth of lymphatic tissue following stress are undoubtedly related to the mechanisms involved in the production of acute involution. There are apparently three mechanisms by which the adrenocortical hormones induce acute lymphatic organ involution. They may produce destruction of lymphocytes (lymphocytolysis), and they may also inhibit homoplastic (mitosis) and heteroplastic proliferation (differentiation) of immature cells (7, 8). In a previous study, differential response of lymph nodes and thymus of adrenalectomized mice to the different types of purified adrenocortical products was conclusively demonstrated and mathematically analyzed. It was then suggested that the lymphocytes in different stages of development display different susceptibilities to the lymphocytolytic and mitotic suppressing effects of the C-11 oxy and 17-hydroxycorticosteroids (8). Thus the mature small lymphocytes, which are the cells most characteristic of the thymus, are more susceptible, and the large lymphocytes, characteristically present in relatively greater numbers in lymph nodes, are more resistant to the lymphocytolytic and mitotic suppressing effect of the adrenocortical steroids (7, 8).

Assuming that the stressing effects of the alcohol intoxication, as induced in this study, stimulated the adrenal cortex to secrete steroids of the C-11 oxy and/or 17-hydroxy type, it would then follow that the greatest involutionary effect would be observed in the thymus, due primarily to the lysis of the small lymphocytes. In contrast to the situation in the thymus, the lymph nodes, with relatively larger numbers of reticulo-endothelial cells, would not display a comparable amount of involution and in some cases would display none following acute stress, depending upon the potency of the endogenous adrenocortical secretions.

The mechanism described above would also lead to an understanding of the manner in which the adrenal cortex may influence the thymic reconstitution. At the time of maximal thymic involution (36 to 48 hours after the alcohol injection) the small lymphocytes of the thymic cortex were greatly reduced in number. At this time, however, there was left a well-defined framework of reticular cells and scattered large lymphocytes distributed throughout the thymic cortex. Thus there was a residue of the more resistant lymphocytes and reticular cells. During the initial rapid growth which followed acute involution, there was an increase in the numbers of reticular cells and immature (reticular) lymphocytes. Mitoses among these cells were commonly observed. During the later stages of reconstituion there was a differentiation of reticular cells and proliferation of lymphocytes. It would thus appear that the reconstitution of the normal thymic structure was a reversal of acute involution. Cytological reconstitution appeared in the order of resistance of cells to the effects of steroid hormones, i.e., the most resistant cells were the first to be restored. Reconstitution of the thymus thus would seem to be associated with a progressive decrease in adrenocortical activity.

If the above were correct, it would then follow that the experimental withdrawal of adrenocortical secretions would accelerate the growth of the thymus, but as it was so clearly demonstrated in this study, a withdrawal of adrenocortical steroids, as induced by adrenalectomy, not only failed to accelerate reconstitution, but induced an additional depression in thymic weights and retarded the onset of growth. We are at present unable to account for the additional drop in thymic weight which occurred following adrenalectomy. However, the growth that eventually occurred was extremely rapid and histologically quite unlike the growth which occurred in the intact-stressed animals. The growth of the thymus which occurred in adrenalectomized animals following the alcohol-induced stress was also quite different from that which has been found to occur following adrenalectomy in non-stressed mice of the same strain and age; where a detectable increase in thymic weight and the maximal increment were found at 24 and 120 hours, respectively, following adrenalectomy (9, 11).

The rapid growth that eventually became established after the 120th hour in the stressed-adrenalectomized animals in the present study, was found to be due to an increase in the number of the small lymphocytes of the thymus. Once this homoplastic growth became established, it proceeded virtually unchecked until the thymic weights exceeded those of the normal non-stressed controls. In contrast, homoplastic growth in the intact-stressed animals decreased progressively as the normal thymic weight was approached. The growth mechanisms thus far described suggest that there exists a fine balance of stimulating and moderating influences which serve to control the rate and degree of growth of lymphatic tissue following a stress-induced acute involution.

The involution and reconstitution of lymphatic tissue has also been demonstrated to be related to nutritional factors (7). Comparison of the body weight loss with the thymic involution in the intact animals suggests that the thymic involution which occurred during the initial 48 hours may well have been partly related to the loss in body weight; however, where the maximal loss in body weight was in the order of 9%, the loss in thymic weight was approximately 70%. Thus the body and organ weight loss were disproportionate. Deprivation of the adrenals induced an additional loss in body weight but despite the cachexia which developed, the thymus grew and exceeded its normal size. Thus in the adrenalectomized animals there was growth of the thymus despite the generalized metabolic disturbances.

Administration of the alcohol subcutaneously, instead of intraperitoneally (6), necessitated doubling the alcohol dose in order to obtain as great an intoxicating and lethal effect. However, on the basis of the thymoregressive response test, the increase in adrenocortical activity was approximately the same as that obtained using the intraperitoneal route. This comparison might at first seem to imply that the response of the pituitary-adrenal axis to a stressful stimulus would follow an "all or none" law. However, since this work was completed it has been conclusively demonstrated that the adrenocortical response to alcohol-induced stress is proportional to the magnitude of the dose (10). The subjective symptoms of intoxication and the mortality obtained using the subcutaneous route paralleled but were delayed compared with those obtained using the intraperitoneal injections. The need for a larger dose using the subcutaneous route was probably related to a slower rate of absorption. It is at present not possible to determine the manner by which either subcutaneous or intraperitoneal injections of alcohol induce the state of stress. The stress response may conceivably be due to central nervous system effects, tissue damage at the site of injection, or general metabolic disturbances associated with liver damage.

The data and interpretations presented in this study are in close agreement with the theories which have thus far been advanced concerning the manner by which the adrenocortical hormones control the mass and cytology of lymphatic tissue (7, 8). It further appears that the reconstitution of the thymus after acute involution is influenced by adrenocortical factors which exert a stimulating effect upon the reticular cells responsible for a rapid early growth. The adrenal cortex then apparently exerts a moderating effect upon the differentiation of reticular cells and proliferation of lymphoid elements. Deprivation of the controlling factors thus not only inhibits the initial reconstitution but also changes the characteristics of the growth which eventually occurs, leading to a hyperplasia of the thymus and lymph nodes.

Acknowledgment

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STUDIES ON THE ASSAY OF ALDOSTERONE: CHROMATOGRAPHY AND CHEMICAL DETERMINATION[1]

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ABSTRACT

Chloroform extracts from urines were prepared for the determination of aldosterone. These were subjected to various types of paper chromatography and the purified extracts evaluated by the ultra violet absorption at 240 mμ and by the blue tetrazolium color reaction. These results were compared with those obtained by bioassay. A sequence of 4 chromatographic systems was considered necessary for most of the urines to obtain a satisfactory agreement between the chemical and the biological determinations. The chromatographic systems were applied in the following sequence: (1) chloroform-formamide; (2) toluene-ethyl acetate-methanol-water; (3) isooctane-tertiary butanol-water and (4) benzene-methanol-water. With this method, aldosterone was isolated from pregnancy urine and identified by the infra red spectra of the isolated material and its lactone.

The comparison between the results of the bioassay and the chemical determination at various stages of the chromatography indicated that some urine extracts contained, even after separation of hydrocortisone, cortisone and corticosterone from aldosterone, one or possibly more substances which caused a lowering of the results obtained by the bioassay. One such compound appears to be separated from aldosterone by development in the E_2B system.

SOME of the problems connected with the extraction of aldosterone from urines have been described in a previous paper (1). In this communication we wish to present some of our experiences during the past four years with the paper chromatographic separation of aldosterone from crude urinary corticosteroid extracts and its chemical determination. It was considered essential to follow the progression of the chromatographic purification of aldosterone from accompanying steroids by bioassay and to compare these results with values obtained by the chemical evaluation methods. A presentation of such experiments was, however, not possible without mentioning an unknown factor, exerting disturbances in the bioassay, which appears to be present in the urinary steroid extracts, especially from pregnancy urines, even after the removal of cortisone, hydrocortisone and corticosterone. In chromatographic polarity, this factor or

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factors appeared to be closely related to aldosterone. In the bio-assay it would depress the sodium retention in the test animals and on some occasions even lead to frank sodium excretion as compared to the control animals. However, no attempt will be made in this communication to precisely describe such a factor, since further work, which is in progress, is required for its identification.

METHODS

Hydrolysis and Extraction: Urine samples were acidified to pH 1.5 and allowed to stand for one or two days at room temperature over a small volume of chloroform. They were extracted three times with chloroform and the extracts were washed five times with one-tenth volume of 0.1N sodium hydroxide and twice with water. The alkali washings were back extracted with chloroform and the combined extracts evaporated to dryness under reduced pressure.

Paper Chromatography: Aldosterone was isolated from the crude corticosteroid residues by various sequences of chromatographic developments on paper as described later.

(a) General Conditions of Chromatography: The chromatographic tanks were kept in a constant temperature room at 78° F. Whatman paper No. 1 was used throughout since it was found to give the least difficulties with background color. The paper, cut in strips 17 cm. in width, was placed in a Soxhlet apparatus and extracted with methanol for 48 hours. A further extraction with chloroform for an additional 48 hours was carried out on the strips to be used for the final run. After application and chromatographic development the position of hydrocortisone, cortisone and aldosterone on the paper strips was determined by their absorption of ultra violet light. The desired zones were outlined and cut at 1 to 2 cm. above and below the absorbing area. The paper was cut into small pieces which were allowed to stand for 3 hours in methanol or ethanol. The eluate was then filtered through a glass fritted filter. To obtain quantitative elution, the paper pieces were shaken with 3 more portions of alcohol and the combined eluates were evaporated under reduced pressure. Methanol and ethanol eluted the steroids equally well, whereas chloroform and methylene dichloride eluted only 13 and 22% of the applied corticosteroid with the same technique.

(b) Chromatographic Systems: The systems of Zaffaroni (2), using toluene-propylene-glycol, benzene-formamide and chloroform-formamide (3), the latter in the modification by Neher and Wettstein (4), were used for the initial runs. These systems were able to accomplish a good separation of the various corticosteroids from relatively large amounts of the crude steroid extract.

To achieve further separation three more systems were used: they were, the system C of Bush (5) (toluene-ethyl acetate-methanol-water 9:1:5:5), the system B_5 of Bush (5) (benzene-methanol-water 14:6:5) and the system E_2B of Eberlein and Bongiovanni (6) (isocotane-tertiary butanol-water 10:5:9).

When highly pigmented or bulky urinary extracts were obtained, a preliminary purification was carried out before the extracts were processed through the separating systems. This was achieved in the n-heptanol-water system of Schmidt and Staudinger (7) using descending paper chromatography instead of an ascending system as originally outlined by these authors. It was found necessary to introduce a beaker with 300 ml. heptanol into the tank, which was filled with 1000 ml. of water, saturated with heptanol, and to prolong the time allowed for equilibration to 3 hours. Applications were made on Whatman Paper No. 1, previously washed with distilled water and dried. The best results were obtained when a crude neutral residue, equivalent to a 15-hour volume of urine, was applied on a paper 17 cm. in width. Twenty-five ml. water satu-

TABLE 1. CONDITIONS AND MIGRATION RATES IN VARIOUS CHROMATOGRAPHIC SYSTEMS

Chromatographic system	TP	CF	E_2B	BuC	BuB_5	HW	BF
Impregnation of paper	propylene-glycol-methanol 1:1	formamide acetone 5:1	—	—	—	—	formamide
Equilibration Time (hrs.)	0	0	5	¾	¾	3	0
Amount of Mobile Phase Used for Development (ml.)	350	25	40	25	25	25	350
Duration of Development (hrs.)	48–72	3.5–4*	18	4–4.5*	4–4.5*	5	5.5
Migration Rate†							
Hydrocortisone (F)	0.470	0.364	0.896	0.632	0.560	1.000	—
Cortisone (E)	1.000	1.000	1.000	1.000	1.000	1.000	0.325
Aldosterone (Al)	0.973	1.010	0.625	0.637	0.857	1.160	—
Aldosterone (monoacetate)	—	1.600	1.820	1.675	2.075	—	—
Cortisone (monoacetate)	—	—	—	—	—	—	0.811
11-dehydrocorticosterone (A)	—	—	—	—	—	—	1.000
11-dehydrocorticosterone (monoacetate)	—	—	—	—	—	—	3.400
Aldosterone (diacetate)	—	—	—	—	—	—	3.360

* Sudan red was added to the standards and papers removed from the tank when the dye had reached the end of the papers.
† With reference to cortisone or 11-dehydrocorticosterone.

rated with heptanol served as the running phase. In contrast with the systems mentioned above, in which an organic solvent is used for the development and in which the urinary pigment and lipid substances are therefore washed over the papers to the solvent front, these materials remain at the starting line in the heptanol water system. In this way they can easily be separated from the corticosteroids which migrate down the paper. Elutates of material remaining at the starting line were repeatedly assayed for a possible occlusion of aldosterone, but none was found under the described conditions. This method has been found to be more advantageous than silica gel columns or petroleum ether-70% alcohol partitions for the purification of urinary extracts.

Details about the development and the running rates of standard corticosteroids in the various chromatographic systems are listed in Table 1.[2]

Physical and Chemical Determinations: The ultra violet absorption of the ethanolic eluate of aldosterone was measured against the eluate from a corresponding strip of blank paper over the range from 200–300 mμ in a Beckman DU Spectrophotometer. The amount of aldosterone present was calculated from the optical density of the absorption maximum:

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[2] For convenience the following abbreviations will be used in the tables and in the text:

Chromatographic Systems:
TP—toluene-propylene glycol system.
BF—benzene-formamide system.
CF—chloroform-formamide system.
BuC—system C of Bush.
BuB_5—system B_5 of Bush.
HW—heptanol-water system of Schmidt and Staudinger.

$$\mu\text{g. steroid/ml.} = \frac{\text{OD max.} \times \text{M} \times 1000}{\text{E}}$$

M = molecular weight (360.4 for aldosterone).
E = molar extinction coefficient (15800 for aldosterone).
λ max. = 238–239 mμ for aldosterone.

The blue tetrazolium (BT) color reaction was performed according to the procedure of Novaczynski *et al.* (8). The reaction was carried out at room temperature and an absorption maximum of the color developed with the BT brand[3] applied was found at 525 mμ. Therefore, this wave length was used for the measurement of the samples instead of 510 mμ. The factors (1/K) for the slopes of the standard curves obtained with cortisone, hydrocortisone and aldosterone were 44.9, 52.2 and 51.6 respectively. With each chemical assay, a standard curve using 5, 10 and 15 μg. of hydrocortisone was dedetermined, against which the samples were evaluated, as the curves for aldosterone and hydrocortisone were found to be almost identical.

For further identification the material was acetylated by the method of Burton *et al.* (2) and the spectra of the sulfuric acid chromogens were determined (9).

Biological Assay: The sodium retaining activity of the urinary extracts was determined by bioassay (10) on adrenalectomized rats receiving a load of 3.5 mg. sodium chloride per rat. One or two groups of animals were injected with desoxycorticosterone acetate (DCA) while the others received urine extracts (in aliquots varying from 10–65 minute volumes per rat). The sodium excretion during the following 4 hours was determined by flame photometry and that of the test animals compared to those of the control and DCA injected animals. For analysis of variance the standard error of the sodium excretion for each animal group was determined. This is listed in Tables 8 and 9 only. In the tables, which present comparisons of results obtained by bioassay and chemical determination in the same patient, the μg. of aldosterone excreted per day are listed with the actual limit of error. These limits of error have been calculated from the standard error of the sodium excretion of each group of test animals and converted into terms of aldosterone.

Standard curves obtained with DCA have shown an index of precision varying from λ = .227 – .260. A standard curve obtained with aldosterone shows an activity approximately 25-fold that of desoxycorticosterone acetate in this assay procedure.

The urinary extracts injected into the test animals for the following experiments were previously purified by various chromatographic procedures as described later.

EXPERIMENTS AND RESULTS

The following experiments were carried out to determine the recovery of aldosterone by bioassay and by the chemical procedure after running the extract in various paper chromatographic systems.

A. *Sequence of Chromatographic Systems*

Urines collected from a healthy pregnant woman in the ante- and postpartum periods were used in this investigation. They were acidified and extracted as described above. Each of the crude neutral extracts was purified by chromatography in the heptanol water system (15 hours urine aliquot per 17 cm. width of paper). The entire paper, with the exception of the pigmented material along the starting line, was eluted and the steroid residue rechromatographed on two sheets in the TP system for $3\frac{1}{2}$ days. The cortisone zone was eluted and reapplied on one sheet for development in the system BuC for 8 hours. A 10-hour aliquot of the eluate from the hydrocortisone region was submitted to bioassay, while the remaining 20-hour aliquot was further chromatographed on strips 3 cm. wide in system E$_2$B for 20 hours. The aldosterone zone was eluted and UV-absorption and the BT-color reaction performed on a 10-hour aliquot. The remaining 10-hour aliquot was used for a final development in the system BuB$_5$ for 7 hours on strips 2 cm. wide. UV-absorption and BT-color reaction were repeated after this step on the aldosterone eluate. The results of this experiment are shown in Table 2.

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[3] Dajac Laboratories, 5000 Longdon Street, Philadelphia 24, Pa.

TABLE 2. ALDOSTERONE ESTIMATIONS AT VARIOUS STAGES OF CHROMATOGRAPHIC PURIFICATION

Normal pregnancy Delivered Sept. 5/57	Aldosterone in μg. per 24 hours				
	(1) After BuC before E$_2$B Bio-assay (limits of error)	(2) After E$_2$B before BuB$_5$		(3) After BuB$_5$	
		UV	BT	UV	BT
Aug. 15-17	12.1 (7.1-17.3)	164	120	125	115
Aug. 18-20	14.2 (9.4-22.2)	235	133	139	135
Aug. 21-23	37.6 (31.6-44.9)	225	147	145	140
Aug. 24-26	42.7 (33.5-54.6)	254	156	165	150
Aug. 27-29	55.2 (45.0-70.9)	266	178	166	169
Sept. 8-10—Postpartum	16.4 (10.9-24.3)	154	28	18	18
Sept. 11-13—Postpartum	10.1 (6.1-17.3)	101	19	11	11

After the samples were chromatographed in system E$_2$B the determinations with the UV-absorption method gave considerably higher values for the aldosterone fraction than the BT-color reaction (Table 2, column 2), thus indicating that the material was still contaminated in spite of the previous 4 chromatographic separations. During the last chromatography in system BuB$_5$, however, this contamination (a UV-positive, BT-negative substance, running a little slower than aldosterone in BuB$_5$ and probably identical with Genest's Compound V) (19) was separated so that at this stage the ultra violet and blue tetrazolium values agreed fairly well (Table 2, column 3) and the measured compound could be considered as being essentially aldosterone. This was further substantiated by the spectrum of the sulfuric acid chromogen of this material, which showed the characteristic peak for aldosterone at 289 mμ. After heating the sulfuric acid solution to 90° C for one hour, maxima at 247 and at 285 mμ were observed as previously described for aldosterone by Wettstein *et al.* (11). The results which were obtained by means of the bioassay before chromatography in system E_2B were grossly at variance with the final results from the chemical estimation during the final period of gestation and only the two postpartum samples showed agreement in the three methods. The lower values obtained by bioassay in this patient suggest that some factor is excreted during pregnancy which appears to interfere with the bioassay of aldosterone. This factor has the same chromatographic behavior as aldosterone in the systems TP and BuC and could be separated from it by systems E_2B and BuB_5. This factor was not present in the two urines collected 10 and 13 days postpartum.

B. Separation of Material Disturbing the Bioassay

Extracts from two urine samples collected from healthy women during their sixth month of pregnancy were chromatographed in systems CF, BuC, E_2B and aliquots for bioassay were removed from the crude extract and after each step of chromatography. Chemical analysis was performed after chromatography in system E_2B. Since agreement between the chemical and biological assays was already reached at this stage, no further development in system BuB_5 was performed (Table 3). The results of the bioassay on the crude extract and the purified fractions from the CF- and BuC paper chromatograms were considerably lower than those obtained after the separation in system E_2B, thus indicating again the presence of a material disturbing the bioassay in the eluates from the CF and BuC chromatograms as well as in the crude extract. In both these specimens the bioassay showed a satisfactory agreement with the chemical determinations after chromatography in the E_2B system. In this respect they behaved differently from the samples in the first experiment (Table 2) which required a subsequent chromatography in system BuB_5 to bring agreement between the UV and BT methods.

TABLE 3. SEPARATION OF BIOASSAY HINDERING MATERIAL

	Fraction	μg. Aldosterone per 24 hrs.		
		UV	BT	Bioassay (limits of error)
Normal Pregnancy case B.M.	crude extract	—	—	8.6 (5.9–14.1)
	CF—eluate	—	—	10.4 (9.3–12.2)
	BuC—eluate	—	—	—
	E_2B—eluate	24.2	24.5	24.7 (15.6–38.0)
Normal Pregnancy case E.S.	crude extract	—	—	37.1 (29.4–44.9)
	CF—eluate	—	—	—
	BuC—eluate	—	—	31.8 (22.8–43.2)
	E_2B—eluate	45.0	45.6	46.6 (37.5–58.8)

C. Background Color from Blank Paper

Since the eluates from blank paper strips showed a certain amount of background color, it was necessary to choose a chromatographic system for the last step of the procedure which would lead to the smallest blank. Paper strips were therefore subjected to various types of chromatography and color reactions on eluates were carried out with blue tetrazolium and with dinitrophenyl-hydrazine. The results, which are shown in Table 4, demonstrate that the system BuB_5 exhibited the lowest blank values with regard to the blue tetrazolium reaction, whereas system E_2B probably is preferable for the dinitrophenyl hydrazine reaction. The same amount of background color was present in extracts whether they were chromatographed in the 4 systems described or only in the last one. From 24 paper blanks handled during the past six months, the optical density of the background color has varied from 0.030 to 0.050 per 8 cm.^{2} paper, when system BuB_5 was used as the last step.

TABLE 4. BACKGROUND COLORS DEVELOPED FROM BLANK PAPER ELUATES

Chromatographic system	OD of background color from 8 cm.^{2} of paper	
	BT	DNPH
HW	0.035	—
C-F	—	0.067
T-P	0.064	—
BuC	0.096	0.060
E_2B	0.272	0.040
E_2B followed by BuC	0.112	—
BuB_5	0.032	0.056
$CF \rightarrow BuC \rightarrow E_2B \rightarrow BuB_5$	0.032	—

D. Recovery of Cortisone, Hydrocortisone and Aldosterone

Since a sequence of 4 chromatographic developments was considered necessary for sufficient purification of the aldosterone fraction in the majority of urines examined, it was important to measure the recovery of the steroids applied on the paper after passing through each system. This was determined by UV absorption and reduction of BT. An average recovery of 95.1% was obtained for cortisone or hydrocortisone when chromatographed in amounts of 75–90 μg. in each of the described systems. This would amount to a total loss of approximately 20% after 4 chromatographic developments. In two experiments, when 60 μg. cortisone were chromatographed consecutively in the 4 systems, CF, BuC, E_2B and BuB_5, recoveries of 81.0 and 84.2% of the initial material were observed with the BT- and 83.8 and 85.9 by the UV-evaluation.

When 24.3, 35.2 and 40.0 μg. aldosterone were applied and chromatographed through these four systems, 75.2–77.9% were recovered as evaluated by the UV and BT method, showing an actual loss of about 25% during the chromatographic procedure.

When the free alcohol of aldosterone was added to water, acidified to pH 1.5, allowed to stand for 48 hours at room temperature and extracted with chloroform in a manner similar to that used for urine, 88% was recovered with the UV- and 85% with the BT-method after chromatography in the system BuC. This demonstrates a loss of about 15% for the hydrolysis, extraction and washing procedure of the aldosterone determination method, including one chromatographic step, so that a total loss of approximately 35–40% would occur during the determination. A similar recovery of 60.8% was obtained when 40 μg. aldosterone were added to the urine before its acidification.

When aldosterone acetate was added to urine samples before acidification, the ester was not quantitatively hydrolyzed under the applied conditions, since only 47.0, 46.5 and 52.8% were recovered after chromatography.

E. Comparison of Aldosterone Values Obtained by Chemical and Biological Assays in Patients with Various Clinical Conditions

Since the four systems, CF, BuC, E_2B and BuB_5 had proven satisfactory when used consecutively for the separation of aldosterone, this combination of chromatographic developments was applied to a series of urines. The eluates from the last chromatogram were subjected to the UV absorption, the BT-color reaction and the bioassay.

TABLE 5. COMPARISON OF ALDOSTERONE LEVELS OBTAINED BY BIOASSAY AND CHEMICAL ESTIMATION AFTER EXTENSIVE CHROMATOGRAPHY

Clinical condition	Aldosterone—μg. per 24 hours		
	UV	BT	Bioassay (limits of error)
Normal males:			
P.S.	2.3	1.8	1.3 (0.9– 1.9)
J.L.	2.6	1.4	2.4 (1.3– 4.0)
M.L.	7.6	7.4	—
J.F.	—	2.1	1.9 (0. – 3.3)
J.F. on ACTH	—	10.9	9.7 (6.4–15.5)
R.M.	—	9.1	8.1 (4.5–14.4)
R.M. on ACTH	—	16.8	12.4 (11.0–14.8)
Normal females:			
I.R.	4.7	1.1	1.2 (0 – 4.9)
O.R.	9.4	7.0	8.1 (6.3–11.2)
D.M.	2.0	1.9	2.2 (1.4– 3.8)
S.A.	4.8	4.2	5.9 (4.2– 7.9)
L.G.	—	5.1	6.7 (3.3–11.2)
L.D.	—	3.8	2.2 (0 – 4.0)
I.P. (1)	—	3.1	3.4 (1.9– 5.9)
I.P. (2)	—	2.4	3.2 (2.1– 4.9)
I.P. (3)	—	3.9	4.2 (2.9– 6.9)
Hypokalemia—J.B.	12.6	14.3	13.2 (11.1–16.1)
Cushing syndrome—L.L.			
untreated	17.0	11.4	12.2 (9.2–17.1)
on ACTH	24.9	24.8	22.2 (19.0–27.1)
post ACTH	8.6	8.5	8.9 (6.6–12.9)
post adrenalectomy	neg.	<1.0	<1.0
Sodium loosing syndrome—R.G.	neg.	<1.0	<1.0
Cushing's syndrome—M.C. untreated	—	5.9	6.3 (4.1– 9.8)

Table 5 shows the results obtained in healthy persons, two patients with Cushing's disease, one case of hypokalemia and a case with a sodium losing condition. In general, fairly good agreement occurred between the chemical methods and the bioassay. In four instances higher levels were obtained by measurement of UV absorption indicating that this determination was the one most sensitive to contaminations.

Table 6 shows aldosterone values obtained during a metabolic study on a case of panhypopituitarism receiving growth hormone preparations. The BT color reaction generally agreed well with the bioassay whereas here again the results of the UV absorption measurements were not so reliable. In the instances indicated by dashes in Table 6, no satisfactory UV absorption curve was obtained. The clinical conditions of this patient were discussed as "case 2" by Beck *et al.* (36).

F. Isolation of Aldosterone from Pregnancy Urine

Thirty-six liters of urine obtained from healthy women in their last month of pregnancy were extracted in the manner described under "Methods.' For purification from pigments the total crude steroid residue was chromatographed in the heptanol-water system using 16 sheets of paper. The brown starting line region was excluded and the rest of the papers eluted. This material was then applied and developed on eight sheets of paper in system CF. From this chromatogram the dark band absorbing light in the region of cortisone, was eluted including 3–4 cm. above its upper borderline, thus excluding the urinary hydrocortisone. A chromatography in system BuC followed using 8 paper strips, most of which showed 4 UV-light absorbing zones. The band in the region of hydrocortisone

TABLE 6. COMPARISON OF ALDOSTERONE LEVELS OBTAINED BY BIOASSAY AND CHEMICAL ESTIMATION AFTER EXTENSIVE CHROMATOGRAPHY IN A CASE OF PANHYPOPITUITARISM (G. L. AGE 18)

Day of	Treatment	Aldosterone—μg. per 24 hours		
		UV	BT	Bioassay (limits of error)
1– 3		3.8	2.7	2.9 (1.9– 4.3)
4– 6		7.2	6.8	7.6 (5.9–10.2)
7– 9		8.1	7.9	7.0 (4.5–10.4)
11–12	Porcine Growth Hormone 10 mg.	6.5	5.5	3.7 (2.7– 5.6)
13–15	Porcine Growth Hormone 40 mg.	4.4	5.0	2.6 (1.6– 4.3)
16–18		2.3	2.7	2.7 (1.8– 4.4)
19–21		6.1	—	5.9 (4.5– 7.2)
22–24		—	—	4.5 (2.3– 8.6)
25–27	Human Growth Hormone 2 mg.	—	10.9	9.6 (7.3–12.1)
28–30	Human Growth Hormone 2 mg.	—	14.0	14.6 (11.9–18.4)
31–33	Human Growth Hormone 5 mg.	(8.0)*	(7.2)*	17.8 (11.3–27.9)
34–36	Human Growth Hormone 10 mg.	9.6	10.2	10.8 (6.9–16.7)
37–39	Human Growth Hormone 20 mg.	15.0	14.1	13.6 (6.8–16.4)
40–42		8.2	7.3	8.0 (5.1–14.7)
43–45		—	6.0	5.3 (4.0– 7.2)
46–48		—	6.5	7.3 (4.5–16.3)
49–51		4.2	4.7	4.0 (3.4– 4.8)

* Some of this material was lost during the procedure.

standard was eluted for development in system E_2B on the same number of papers. Three zones were observed, the center one corresponded to the position of aldosterone. This was eluted and rechromatographed in system BuB_5, which did not separate any visible amounts of UV light absorbing compounds (Genest's Cpd. V) and in this behaved differently from the urine used in experiment A (Table 2) but similar to the two pregnancy urines in experiment B which also showed no evidence for the presence of Cpd. V. The aldosterone region showed a material which absorbed UV light maximally at 239 mμ. The amounts of this material were estimated by the three available methods and are shown in Table 7. The results determined by bioassay for aldosterone activity, increased with progressive purification and reached the levels measured by the BT-reaction after the last chromatography, thus indicating again, for the crude fractions, an inhibition of the aldosterone effect in the test animals by some contaminating material, some of which was removed by development in the E_2B-system, whereas a remainder was separated out by BuB_5. An infra red spectrum of the isolated material was obtained through the kindness of Dr. Lieberman and Dr. Ulick. We are also indebted to them for the preparation of the lactone 874 of Reichstein (12) from our material and for the infrared spectrum of the latter substance. Both spectra were considered compatible with the ones of authentic aldosterone and of lactone 874 respectively. When the lactone prepared from the urinary material was chromatographed in cyclo-hexane-benzene (1:1)/formamide with the authentic compound for 6½ hours, both showed the same running rate (lactones 19.25 cm., adrenosterone 22.25 cm.).

TABLE 7. ALDOSTERONE ISOLATED FROM PREGNANCY URINE

System	Aldosterone in μg.		
	UV	BT	Bio-assay (limits of error)
After BuC	790	3660	328 (267–396)
After E_2B	518	760	414 (337–521)
After BuB_5	478	480	468 (397–569)

An acetate was prepared from material isolated in a similar manner from other batches of pregnancy urine. This substance showed, in system BF, the running rate expected for aldosterone diacetate (Table 1). The sulfuric acid chromogen spectrum had a single maximum at 289 mμ and exhibited peaks at 247 and 285 after heating to 90° C, which added further evidence for the identity of the isolated material with aldosterone.

DISCUSSION

The necessity for a purification of the crude neutral corticosteroid extracts had already been recognized at the time when only bioassay methods were available for the determination of aldosterone. Hydrocortisone, cortisone, corticosterone, which are known to occur in these extracts, may affect the excretion of sodium and/or potassium in adrenalectomized animals. In our assay procedure cortisone was found to lower the aldosterone results when present in amounts of 25 μg. or more per rat; 1–10 μg. had little effect on the results (Table 8). Hydrocortisone, when injected directly into test animals, showed a sodium excreting effect at dose levels from 30 to 50 μg. per rat, whereas lower levels did not appear to have any significant influence. Corticosterone also produced some sodium excretion at levels of 5–15 μg. per rat, but did not alter the sodium excretion at 20 μg. per rat. Luetscher and Johnson (13) found that hydrocortisone, cortisone, corticosterone, as well as 11-dehydrocorticosterone, had an excreting effect in their assay procedure and therefore chromatographed the crude urinary extracts in the TP and/or the BuB_5 system before submitting them to bioassay. The observations of various authors with regard to the sodium retaining or sodium excreting activity of cortisone and hydrocortisone at various dose levels in their respective assay methods, have been summarized by Streeten *et al.* (14).

TABLE 8. INFLUENCE OF ADDED CORTISONE ON THE ALDOSTERONE RESULTS OF THE BIO-ASSAY ON A PREGNANCY URINE

Urine sample Min. vol./rat	Cortisone added μg./rat	Na-excretion Meq. with standard error	Aldosterone μg./24 hrs.
10	0	.139± .016	7.00
10	1	.140± .012	6.85
10	5	.141± .013	6.60
10	10	.141± .008	6.60
10	25	.172± .015	3.10

The purification of the aldosterone fraction from crude extracts is even more of a necessity when the hormone is measured chemically, since the available evaluation methods are group reactions, the formazan color measuring the α-ketol group in the sidechain, the ultra violet absorption the Δ^4-3-keto group in ring A, and the formation of dinitrophenyl hydrazones the keto groups in general. Only the latter reaction may, according to Gornall and Gwilliam (15), be considered at least partially specific for aldosterone, since conditions can be chosen which lead to an absorption maximum of 460 mμ for the aldosterone derivative, while most other steroids show, with the same treatment, maxima at 495 mμ.

Simpson and Tait (16) were the first to demonstrate an effective separation of aldosterone from cortisone and hydrocortisone by the use of the two paper chromatographic systems TP and BuC. Subsequently, Neher and Wettstein (17), developed a physico-chemical method for the measurement of aldosterone in urine, based upon these procedures. A preliminary purification of the chloroform extract with a partition between petroleum ether and aqueous ethanol, followed by a column chromatography on silica gel, were carried out. The eluates from the BuC chromatograms were evaluated by a semi quantitative method, which compares the intensity of the BT-color and soda fluorescence of the aldosterone fraction with hydrocortisone. In the following year the authors reported an improvement in their procedure (4) and replaced the TP by the CF system, which required only 4 hours running time instead of 3 days. They also mention in this article the possibility that in such eluates, which are free of cortisone, hydrocortisone and corticosterone, other factors might occur.

More recently, Novaczynski, Genest *et al.* (18, 19) showed that other compounds actually were present in the aldosterone region of the BuC chromatogram and that more than two chromatographic developments were needed for a complete separation of aldosterone from these substances. So far they have characterized six compounds, in addition to aldosterone, from urines of patients with Cushing's disease and from that of healthy pregnant women. They reported that compounds III and V occur most frequently. Compound III was found to be increased in patients on high intakes of potassium (19) and compound II is believed to be the 11-keto analogue of aldosterone. To separate all these compounds completely from aldosterone the authors suggested, after purification of the extracts on a silica-gel column, the use of at least 3 chromatographic systems in the following sequence: ethylene-glycol-toluene, E_2B and BuB_5.

Gornall and coworkers (15, 20, 21) also suggested that more than two chromatograms are needed to obtain a pure aldosterone fraction and proposed a sequence of two modified BuB_5 systems (addition of 5 and 10% ethylacetate to the benzene) and the TP system.

Hernando and coworkers (22) observed that the eluate of the aldosterone zone, obtained after purification in 3 systems, the florisil column and the TP and BuC systems of paper chromatography, divided into 3 zones when run in the E_2B system. These were aldosterone, compounds III and /or IV of Genest and another compound more polar which reduced BT. In our own experiments, we have observed compounds III or IV separating from aldosterone in the E_2B system in most of the urines as a UV light absorbing, BT not reducing material, traveling a little faster than cortisone. Compound V was observed from the pregnancy urine of experiment A, when it was separated by the BuB_5 system. An additional compound (BT positive and UV negative), not described by Novaczynski *et al.* (18,19) was observed to be separated in the final BuB_5 system from the pregnancy urine of experiment F (Table 7). After its removal the bioassay showed higher results.

These various observations indicate that there are several compounds present in urine with running rates on paper similar to aldosterone. These do not occur consistently in all urines and are often present in only trace amounts. To a great extent these are derived from the dark absorbing zone seen directly below the cortisone region on the initial papers run in the CF system. This zone is excluded when the Neher and Wettstein method is followed precisely. In practice, however, and when the TP system is applied, the cortisone zone and this zone often overlap and it is difficult to obtain a clear-cut separation; therefore, it is felt to be safer to include the upper part of this dark zone and rely on the subsequent systems for a more complete separation of aldosterone.

Another sequence of chromatographic separations has been suggested by Moolenaar (23, 24) who used toluene-octanol-methanol and water in two systems with different component ratios to separate aldosterone. A modified method of Gornall was used for the final evaluation.

All these assays are sensitive to approximately 1 μg. of aldosterone. The recovery of added aldosterone from urine varies as follows, Neher and Wettstein (4) 75%; Novaczynski *et al.* (19) 83%; and Hernando (22) 20–31%. Our recovery is around 60%.

Lately more sensitive methods have been developed with the help of isotopes, which will allow aldosterone determinations in blood. Ayres and coworkers (25), using column chromatography on Kieselgur were able to measure aldosterone diacetate-C_{14} in the range from 0.2 to 4.0 μg. with great precision. Kliman and Peterson (26), labeling the hormone with tritiated acetate, reported a sensitivity of 1/100 μg. of aldosterone.

When the results of the chemical assay were compared with those of the bioassay, a generally good agreement was observed when the samples were extensively purified (Tables 3, 5 and 6). Lower values were often obtained by bioassay from crude extracts and from eluates after preliminary chromatography, than from the chemical determination as shown in Tables 2 and 3. Such differences may be primarily due to the interfering action of hydrocortisone, cortisone or corticosterone for all assays done on the crude extracts. Differences remaining after chromatography in the systems CF and BuC must, however, be interpreted as a contamination of the aldosterone fraction with other substances, either actively sodium excreting or aldosterone antagonizing, which have running rates similar to this hormone in the TP, CF and BuC systems (see Experiments, A, B and F). Tables 3 and 7 indicate that one of such materials can be separated in the E_2B system; whether this is identical with Compound III of Novaczynski *et al.* (35) is not established at the present time. Another one seems to require the BuB_5 system (Table 7).

The possibility of the existence of material with such biological effects was suggested to us by several observations during our experience with the bioassay in the past three years. An extract, roughly purified in the BuB_5 system for the purpose of removing compounds F, B, and most of Compound E, was frequently found to show less aldosterone when assayed at a higher dose level than at a lower dose level (Table 9). That this effect could not be explained on the basis of traces of cortisone which had not been completely removed is evident from the data presented in Table 8. The biological action of the material affecting the bioassay of aldosterone exerted its influence only in higher concentration, and could be diluted out to a certain degree. Other evidence for the presence of sodium excreting substances was observed in certain clinical conditions characterized by a sudden active output of sodium in the patient. The extract of such urines, when injected into adrenalectomized animals, actually increased the sodium output above that of the control group. This effect was observed in a patient with liver disease at a time when the ascites was spontaneously being resorbed and sodium diuresis was occurring (27). Occasionally this effect is observed after ACTH withdrawal in normal individuals or in patients when the injected extract caused a significant excretion of sodium in the test animal. Certain urines from pregnant women also appear to contain such a factor (Table 9).

TABLE 9. EXAMPLES OF URINE EXTRACTS, PURIFIED BY BuB_5 CHROMATOGRAPHY, WHICH SHOW A GREATER INTERFERENCE IN THE BIOASSAY ON HIGHER DOSE LEVELS OF INJECTION

Case	Clinical Condition	Aldosterone by Bioassay in µg /24 hours*			
		10 min †	*15 min* †	*30 min* †	*45 min* †
N.L. (1)	Normal Pregnancy	9 1 (149± 017)‡		138%ex (265± 023)‡	
(2)		15 6 (123± 022)		3 9 (136± 015)	
(3)		26 8 (098± .015)		9 8 (094± 017)	
J S	Normal Pregnancy		5 1 (173± .013)		132%ex. (297± 027)
S B. (1)	Toxemia of Pregnancy		16 3 (114± 021)		7 3 (098± 009)
(2)			26 5 (089± 014)		10 0 (082± 013)
(3)			39 1 (068± 010)		11 5 (075± 016)
(4)			57 6 (047± 008)		14 4 (061± 006)
K M. (1)	Toxemia of Pregnancy	13 1 (100± .014)§		7 2 (082± 012)§	
(2)		15 2 (094± 011)		3 0 (113± 015)	
(3)		34 0 (066± 008)		6 8 (084± 007)	
J.K (1)	Toxemia of Pregnancy	9 1 (134± 008)		3 1 (133± 016)	
(2)		13 4 (118± 007)		3 3 (131± 011)	
G Z	Toxemia of Pregnancy	24 5 (110± 017)		12 5 (088± 019)	
G J.	Toxemia of Pregnancy	10 3 (156± 021)		3 3 (159± 022)	

* The figures listed in parentheses indicate the sodium excretion of the test animals in m.eq. with the standard error
† Minute volume injected per rat
‡ Sodium excretion of the control group 193± .024 m eq.
§ Sodium excretion of the control group 225± 019 m eq.

Similar observations have been mentioned by Neher and Wettstein (4) who obtained more crystalline aldosterone from the urines of patients with nephrosis and with cardiac failure than the biosassy had previously indicated. Since this material which is affecting the bioassay is chromatographically so closely related to aldosterone, one might speculate that it could be derived from the hormone during the procedure, possibly by oxidation. In this connection it is interesting to note that recently Wettstein (28) reported on the sodium excreting properties of Reichstein's compound No. 885 (34), *which is the lactone of an aldosterone analogue carrying a* carboxyl group in the C_{18} position.

The identification of the urinary sodium retaining material with aldosterone was first established by Luetscher, Neher and coworkers in nephrosis (29, 30) and later in cardiac failure (31). Aldosterone was also chemically identified in the urine of patients undergoing surgery (32) and in the urine of pregnant women by Novacznyski *et al.* (18). In our own studies the characterization from pregnancy urine is further substantiated by the infra red spectrum and acetate formation of the hormone obtained from pregnancy urine.

The assay of aldosterone either by chemical or biological procedures is still a time-consuming operation. Only a small fraction of the amount of hormone secreted by the gland appears in the urine as free steroid. The recent report of Ulick and Lieberman (33) of the isolation of a tetra-hydro derivative of aldosterone present in much larger quantities than aldosterone in urine offers another means of studying the metabolism of this hormone in clinical conditions.

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EFFECTS OF ACUTE AND CHRONIC TREATMENTS WITH CARBUTAMIDE (BZ-55) ON OBESE-HYPERGLYCEMIC MICE AND THEIR LEAN LITTERMATES[1]

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ABSTRACT

The acute administration of carbutamide was found to have a hypoglycemic effect in fasted normal mice, while it had no definite effect on the already low blood sugar levels of fasted obese hyperglycemic mice. Chronic oral treatment with carbutamide did not modify the food intake and weight increases of either type of mice during the seven weeks treatment. In obese-hyperglycemic mice no improvement of the "diabetes" was observed, both in fed and fasted states. Lean littermates, whether fasted or fed, showed a reversal of the direction of effect of a single dose. These animals were, rather paradoxically, rendered paradiabetic after four weeks of treatment, and responded to glucagon and growth hormone with a reaction similar to that seen in untreated obese-hyperglycemic mice.

THE hypoglycemic action of carbutamide (BZ-55) and related sulfonylureas is said to take place only in the presence of insulin. Several hypotheses have been advanced to explain this effect: carbutamide may promote increased insulin secretion or it may potentiate the action of insulin, this in turn either at the hepatic level or/and in extrahepatic tissues (1, 2). It has also been suggested that sulfonylureas act by decreasing hepatic phosphorylase activity (3, 4). The availability of mice with the hereditary obese-hyperglycemic syndrome seemed to offer an opportunity to elaborate the mechanism of action of carbutamide. These animals, besides showing obesity and a relatively insulin resistant type of hyperglycemia (5) exhibit an increase in the insulin content of their pancreas (6) and an increased hepatic phosphorylase activity (7). Obese-hyperglycemic mice seem therefore particularly suitable for the testing of hypothesis relating the effect of carbutamide to the potentiation of insulin or to decrease in hepatic phosphorylase activity. It appeared useful to use carbutamide both in acute and in chronic treatments on both obese-hyperglycemic mice and their normal littermates.

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MATERIAL AND METHODS

25 obese-hyperglycemic and 27 littermates were kept in individual cages on ground Purina chow. As nearly as possible equal numbers of males and females were used in each group. As the duration and development of the hyperglycemic may influence the response to carbutamide, mice of 2 age ranges were distributed among the groups in the proportion of 2 young (2 months old) to 1 adult (4 months old) (see Table 1).

TABLE 1. CHRONIC TREATMENT WITH CARBUTAMIDE

Average daily food intake and final weight increase (in percentage from the starting values) of obese-hyperglycemic mice and their lean littermates. Comparison between control animals and animals treated orally with carbutamide.

	Untreated			Treated with carbutamide		
	No.	Average daily food intake, gm.	Weight increase	No.	Average daily food intake, gm.	Weight increase
Littermates:						
Young	9	5.3	16	9	4.9	14
Adult	4	5.1	0	5	6.0	5
Total	13	5.2 ± 1.4		14	5.2 ± 1.4	
Obese:						
Young	9	6.3	35	9	5.9	32
Adult	4	6.5	16	3	6.6	9
Total	13	6.4 ± 1.4		12	6.1 ± 1.2	

A. Chronic experiment

After a control period of 3 weeks, 12 obese and 14 littermates received carbutamide uniformly mixed with the ground chow (2.5 gm. carbutamide per Kg. of diet) while the remaining (control) animals were maintained on unmodified diet. During the period of 10 weeks throughout the experiment, the following records and determinations were made:

1. the food intake, once a week (corrected for spilling, which occurred to a larger extent in lean littermates than in the obese)
2. the postprandial weight of each mouse, one to three times a week
3. the postprandial blood sugar on days 1, 28, 35, 42, 49, and 70 (approximately at 10 A.M. every day)
4. the fasting blood sugar after 17 hours on days 2, 36, and 56.

In addition, the effects of growth hormone and HGF were studied in the following manner:

Bovine growth hormone (Armour list 916, potency 106%, contamination with ACTH less than 6×10^{-5}U.S.P./mg., with TSH less than 8×10^{-3} U.S.P./mg. and Prolactin less than 10^{-1} I.U./mg.) was dissolved in distilled water, and 1 mg. injected subcutaneously on the 42nd day. The blood sugars were checked immediately before and 17 hours after the injection, on fed animals.

HGF (crystalline Lilly CT 871, with an insulin content of less than 5.10^{-2} U./mg.) was dissolved in a phenol solution (pH 2.5) to a final strength of 40 μg./ml. 10μg. were injected intraperitoneally in each fed mouse on the 49th day. The blood sugars were determined immediately before and 20 minutes after the injection.

B. Acute experiment

The acute effect of carbutamide was studied on the same control and treated animals fasted for 17 hours, on the 56th day of the chronic experiment. The carbutamide was dissolved in a NaOH solution, pH 8.0 and 12.5 mg. of the sulfonylurea injected subcutaneously into each mouse. The blood sugar was determined before and one hour after the injection.

Blood was collected from the tail vein and determinations were carried out on 0.05 ml. samples by the method of Somogyi-Nelson (8, 9) (filter 66 for low readings and filter 54 for high readings). Prior to the sampling, the mice were lightly anesthetized (10 mg. nembutal/100 gm. body weight).

RESULTS

A. Chronic experiment

No trend in intake during the course of the experiment having been discovered, the data in each group of mice were pooled and compared (Table 1). Carbutamide, when mixed with the food did not modify the food intake of obese mice and their littermates, whether young or adult. The relative hyperphagia of obese-hyperglycemic mice (6.4 gm. daily instead of 5.2 gm. for the littermates in this series) persisted. The weight increases are graphically recorded in Figure 1 and expressed in percentage from the starting values in Table 1. Again there was no difference between treated and untreated animals. Young obese hyperglycemic mice gain

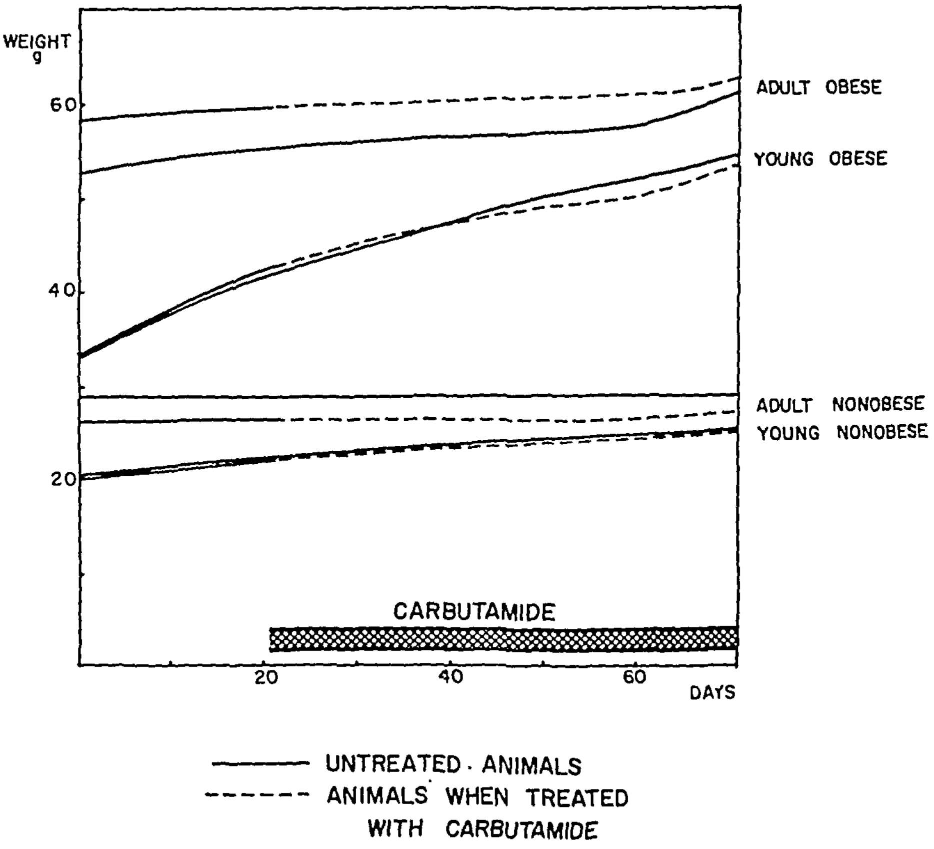

FIG. 1. Average weight curves of adult and young obese-hyperglycemic mice and their non-obese littermates. After a control period of 3 weeks, half of the animals in each group received carbutamide mixed with the chow (2.5 gm. per Kg. of diet) while the remaining mice were maintained on the unmodified diet.

TABLE 2. EFFECT OF A CHRONIC TREATMENT WITH CARBUTAMIDE ON THE BLOOD SUGAR LEVELS OF OBESE-HYPERGLYCEMIC MICE AND THEIR LEAN LITTERMATES

(Values are given in mg. per 100 ml.)

	Dietary status	Day of experiment	Blood glucose of untreated young and adult mice			Day of treatment‡	Blood glucose of carbutamide-treated young and adult mice		
			Young (9)*	Adult (4)	Young and adult†		Young (9)	Adult (5)	Young and adult†
Littermates	Fed	1	108	86	101 ± 23	—	98	98	98 ± 21
		28	90	104	94 ± 16	7	109	113	110 ± 29
		35	125	131	127 ± 19	14	152	139	147 ± 42
		42	108	113	112 ± 24	21	130	124	128 ± 19
		49	94	95	94 ± 21	28	146	141	152 ± 51^{xx}
		70	89	89	89 ± 21	49	112	133	120 ± 31^{xx}
	Fasted (17 hrs.)	2	41	58	46 ± 39	—	42	53	46 ± 33
		36	53	82	62 ± 35	15	85	75	81 ± 27
		56	64	55	62 ± 21	35	92	84	89 ± 23^{xx}
	Dietary status	Day of experiment	Blood glucose of untreated young and adult mice			Day of treatment	Blood glucose of carbutamide-treated young and adult mice		
			Young (9)	Adult (4)	Young and adult†		Young (9)	Adult (3)	Young and adult†
Obese	Fed	1	212	—	—	—	220	275	237 ± 55
		28	211	243	221 ± 84	7	204	264	219 ± 75
		35	231	195	220 ± 111	14	178	235	192 ± 96
		42	245	192	229 ± 133	21	210	221	213 ± 128
		49	233	180	217 ± 118	28	207	150	197 ± 81
		70	236	193	223 ± 118	49	202	204	203 ± 57
	Fasted (17 hrs.)	2	79	67	75 ± 28	—	92	72	87 ± 51
		36	66	96	75 ± 43	15	112	118	117 ± 51^{x}
		56	25	33	28 ± 32	35	81	115	91 ± 43^{xx}

* Numbers in parentheses are numbers of animals.

† There was no significant difference between the blood sugar levels of young and adult mice. The standard deviations of the average blood levels are therefore only given for the combined data.

‡ The animals receiving carbutamide were treated orally with the drug from the 21st day of the experiment.

^{x} and ^{xx}: differences between treated groups and control groups significant at 5% and 1% levels respectively.

weight rapidly in both treated and untreated groups, while the weight of the littermates increased slowly.

The chronic oral treatment with carbutamide increased the blood sugar level of the lean mice (Table 2). After 28 days of treatment this increase was significant both in the fed and in the fasting states. After the same duration of treatment the hyperglycemia of obese-hyperglycemic mice was not lessened. The slight decrease of postprandial sugar levels was not significant. Moreover, the chronic treatment appeared to decrease the extent of blood sugar fall resulting from fasting.

Growth hormone administered to control animals fed *ad libitum* decreased blood sugar when it was measured 17 hours after administration to mice which did not receive carbutamide (Table 3). This effect was not observed on lean animals who had received carbutamide in their diet for 43 days. In obese-hyperglycemic mice the slight hyperglycemic effect of growth hormone (10) was enhanced by carbutamide treatment. As expected (5) the observed hyperglycemic effect of an intraperitoneal injection of 10 μg. of glucagon was greater in the obese than in the lean group (Table 3). Carbutamide increased the hyperglycemic response. This increase was significant in the lean group, not significant in the obese group.

B. Acute experiment

The subcutaneous administration of 12.5 mg. of carbutamide to fasted

TABLE 3. BLOOD GLUCOSE LEVELS OF OBESE-HYPERGLYCEMIC MICE AND CONTROLS UNTREATED OR CHRONICALLY TREATED WITH CARBUTAMIDE WHEN SUBJECTED TO INJECTION OF GROWTH HORMONE, GLUCAGON AND CARBUTAMIDE

Type of animal and number	Chronic treatment	Acute treatment	Day from start of experiment	Dietary status	Blood glucose, mg. per cent			Effect of treatment on blood glucose and significance	
					Before treatment	After treatment	Change	Difference, mg. per cent	Significance
Littermates (13)	None	Growth Hormone 1 mg. subcutaneously	42	Fed	112 ± 24	74 ± 21	−38 ± 34	44 ± 31	$p < 0.01$
(14)	.25% Carbutamide in diet	Growth Hormone 1 mg. subcutaneously	42	Fed	128 ± 19	134 ± 31	6 ± 28		
(13)	None	Glucagon 10 μg. intraperitoneally	49	Fed	94 ± 21	129 ± 43	35 ± 41	71 ± 52	$p < 0.01$
(14)	.25% Carbutamide in diet	Glucagon 10 μg. intraperitoneally	49	Fed	152 ± 51	257 ± 71	106 ± 62		
(13)	None	Carbutamide 12.5 mg. subcutaneously	56	Fasted	62 ± 21	23 ± 19	−39 ± 25	43 ± 27	$p < 0.01$
(14)	.25% Carbutamide in diet	Carbutamide 12.5 mg. subcutaneously	56	Fasted	89 ± 23	93 ± 32	4 ± 28		
Obese (13)	None	Growth Hormone 1 mg. subcutaneously	42	Fed	229 ± 133	237 ± 110	9 ± 65	16 ± 82	$p < 0.5$
(12)	.25% Carbutamide in diet	Growth Hormone 1 mg. subcutaneously	42	Fed	213 ± 128	236 ± 106	25 ± 100		
(13)	None	Glucagon 10 μg. intraperitoneally	49	Fed	217 ± 118	343 ± 117	126 ± 78	51 ± 66	$p < 0.1$
(12)	.25% Carbutamide in diet	Glucagon 10 μg. intraperitoneally	49	Fed	197 ± 81	374 ± 87	177 ± 47		
(13)	None	Carbutamide 12.5 mg. subcutaneously	56	Fasted	28 ± 32	52 ± 55	24 ± 34	38 ± 36	$p < 0.02$
(12)	.25% Carbutamide	Carbutamide 12.5 mg. subcutaneously	56	Fasted	91 ± 43	77 ± 60	−14 ± 36		

lean mice receiving the drug for the first time, lowered significantly the blood glucose level (Table 3). Animals treated orally for 56 days with carbutamide no longer showed this effect. In obese-hyperglycemic mice, not previously treated with oral carbutamide there was some inconsistent increase in blood glucose after an injection. In chronically treated animals there was practically no modification of blood glucose.

DISCUSSION

The daily consumption of carbutamide during the chronic experiment averaged 0.50 gm./Kg. of body weight in the lean animals, and 0.30 gm./Kg. in the obese. These daily oral doses were approximately equal to those given subcutaneously in the acute experiment. Although these doses are large on a ratio-to-body-weight basis, when compared with the dosages given to human patients, they have no deleterious effects on the appetite or on the weight of young and adult animals.

The *acute administration* of carbutamide was found to be hypoglycemic in fasted lean mice. This is similar to results obtained in normal animals of other species. Nevertheless, it was without effect on fasted obese-hyperglycemic mice. These mice have hypertrophied β cells and a pancreas with a high insulin content (6). Several authors have indicated their belief that insulin is necessary for the action of carbutamide, the evidence presented here demonstrates that presence of this hormone in the pancreas does not necessarily guarantee a successful therapy. However, this observation does not rule out the hypothesis that carbutamide acts to release insulin from the pancreas. Since it has been shown that the hyperglycemia of these mice responds only slightly to moderate doses of exogenous insulin (6), endogenous hypersecretion may also be relatively ineffective. A more likely alternative is that the pancreas of obese-hyperglycemic mice already produces and releases all the insulin it is capable of so that additional stimulation is without effect. The hypoglycemia observed in lean mice after the first single subcutaneous dose of carbutamide is not observed in the group previously subjected to oral treatment with the drug. This suggests that some adaptation to the treatment takes place. Indeed the slight hyperglycemia induced in chronically treated lean mice, whether fasted or fed, shows a reversal of the direction of effect of a single dose. Finally, and rather paradoxically, lean mice on the chronic treatment are rendered paradiabetic, while the condition of the obese-hyperglycemic remain unchanged. Lean mice, chronically treated with carbutamide respond to glucagon and growth hormone with a reaction similar to that seen in untreated obese-hyperglycemic mice (5, 10). Chronic administration of carbutamide enhances the hyperglycemic response to glucagon, a fact perhaps related to increased liver glycogen following treatment with sulfonylureas (11, 12) (but in apparent contradiction with results obtained *in vitro* (3, 4)). Carbutamide treatment also eliminates in these non-obese mice the hypoglycemia occurring after growth hormone injection.

An increased food consumption is not involved in the development of this paradiabetic condition in normal mice. An enhanced neoglucogenesis seems unlikely since a normal pancreas should be able to control the hyperglycemia due to neoglucogenesis. There remains the possibility of decreased glucose utilization. In order to explain this decreased glucose utilization, one can hypothesize that a pancreatic exhaustion occurs secondary to a period of hyperactivity. An alternative hypothesis is that in mice as in rats (13, 14) and in dogs (15) the insulin sensitivity decreases during the chronic treatment with carbutamide.

Comparison of data obtained here in mice with data obtained in other animal species indicates that chronic treatment with carbutamide has effects which are functions of species, dose and duration of treatment. In fasted dogs, the hypoglycemic effect is still observed and the rate of glucose utilization enhanced after several weeks of treatment (15). In fasted rats, hypoglycemic effect and improved glucose utilization (13) fade concomitantly. After two weeks of treatment, the rate of glucose utilization is decreased (14), this decrease still persists after 3 months (16). Conflicting results have been obtained in studies of oral glucose tolerance in both species subjected to chronic treatment with sulfonylureas (17, 18, 19). It now appears that the mouse shows yet a different type of reaction: in obese-hyperglycemic mice, we observe essentially no improvement of the "diabetic" condition; in normal mice there is a slowly developing tendency toward a paradiabetic condition.

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INFLUENCE OF GONADAL HORMONES ON THE METABOLISM OF CORTISONE

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ABSTRACT

Liver homogenates from adult male rats reduced the 17,21-dihydroxy-20-ketone sidechain of cortisone more rapidly than did similar preparations from female rats. Conversely, the female preparation metabolized the $\Delta 4$–3 ketone group at a faster rate than did the male. The ratio A-ring reduction/sidechain reduction for the female is 3.4; for the male it is 0.88. The sex difference in the metabolism of the sidechain of cortisone is not present in rats younger than about 25 days of age but becomes evident by the time the animals are 30 days old. Gonadectomy and injections of estradiol-17β and testosterone indicate that the male hormone promotes the ability of the liver to reduce the sidechain of cortisone while the female hormone has an inhibitory effect.

IN THE course of the metabolism of adrenocortical hormones reduction of the C-20 and $\Delta 4$–3 ketone groups is known to occur. These reactions occur in man (1) and in various laboratory animals; can be demonstrated both in the intact animal and in liver slices, homogenates and subcellular fractions (2, 3, 4, 5). Enzymes involved in these conversions have been characterized as to intracellular localization (5), substrate specificity (3, 4), and cofactor requirements (5, 6, 7). The effects of various endocrine factors on the reduction of the A-ring and sidechain of adrenocortical steroids have been studied. Brown and co-workers (8) have reported pretreatment of rats with cortisone or triiodothyronine increases the ability of liver slices to metabolize hydrocortisone while hypophysectomy or thyroidectomy reduces the rate of metabolism. Likewise, Yates et al. (9) and McGuire (10) have found thyroid hormones to increase the rate of reduction in ring A of adrenocortical steroids by liver slices and homogenates. This paper reports effects of gonadal hormones on the enzyme systems of the liver responsible for reduction of the C-20 and $\Delta 4$–3 ketone groups of cortisone. A preliminary report on this subject has appeared (11).

METHODS

Rats from the Holtzman strain were used throughout this study. The selected rat was killed by cervical section and exsanguinated. A portion of the liver was rapidly excised, chilled and homogenized in 0.25 M sucrose made 0.04 M with nicotinamide. Homogenization was performed at 0° C in a Potter homogenizer with a Teflon pestle. Four ml. of whole homogenate containing 200 mg. tissue were incubated with 150 μg of cortisone added in 0.1 ml. ethanol. Incubation was for 2 hours at 37° C in an atmos-

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phere of nitrogen. The pH was maintained at 7.4 with 0.1 ml. of molar potassium phosphate buffer. The TPN system consisted of 0.01 ml. of 0.3 *M* manganese chloride, 0.1 ml. of 0.2 *M* sodium isocitrate and 0.4 ml. of 10^{-4} *M* TPN. The addition of isocitric dehyrogenase was found to be unnecessary for maximal TPN effect. However, it is noted that whole homogenates were used in these experiments and endogenous isocitric dehydrogenase was doubtlessly present in the system.

Following incubation the homogenate was extracted with 20 ml. chloroform. The chloroform layer was washed with 0.1N NaOH and 0.1N HCl and a 1 ml. aliquot evaporated to dryness. Alteration of the sidechain of cortisone was followed by the Porter-Silber reaction (12) after the dry residue had been taken up in 1 ml. of methanol. Reduction of the 3-keto $-{}_{\Delta}4$ function in ring A was determined by adding 4 ml. of methylene chloride to the dried aliquot and reading the resulting solution in a Beckman DU spectrophotometer at 240 mμ. In each experiment a duplicate, unincubated control flask was prepared, to which chloroform was added immediately after the addition of cortisone. The data were analyzed by the small sample t test and mean differences were considered significant if the value of P was 0.01 or less.

RESULTS

The results presented in Table 1 indicate that liver homogenates from adult male rats reduce the sidechain of cortisone at a faster rate than do the corresponding preparations from female rats. Addition of the TPN system produced an increased rate of cortisone reduction by both male and female preparations. The increase was relatively greater with female liver but the marked sex difference is still apparent. The disappearance rates of the ${}_{\Delta}4$–3 ketone position of cortisone, incubated in the presence and absence of the TPN system, are also shown in Table 1. In this instance the situation is the reverse of that seen with respect to sidechain metabolism in that the female reduces the A-ring more rapidly than does the male. This is apparent whether or not TPN is added to the system.

Various experiments were performed to elucidate further the nature of the observed sex difference in the metabolism of cortisone. Information on A-ring changes is still incomplete and for this reason only observations on

TABLE 1. THE METABOLISM OF CORTISONE BY LIVER HOMOGENATES AS MEASURED BY THE PORTER-SILBER REACTION AND ULTRA-VIOLET ABSORPTION AT 240 mμ. THE SEX DIFFERENCE AND EFFECT OF TPN ARE SHOWN

	No. of animals	Micrograms of cortisone lost				E 240 mμ/Porter-Silber with added TPN system
		Porter-Silber reaction		E 240 mμ		
		Without added TPN system	With added TPN system	Without added TPN system	With added TPN system	
Normal males (300–350 gm.)	17	72 ± 2	104 ± 5	41 ± 5	92 ± 2	0.88
Normal females (220–240 gm.)	16	12 ± 2	32 ± 2	60 ± 3	109 ± 2	3.4

Results in this and in the following table are given as means ± S.E.

TABLE 2. THE EFFECT OF GONADECTOMY AND OF GONADAL HORMONES ON THE RATE OF REDUCTION OF THE 17-21-DIHYDROXY-20-KETONE SIDECHAIN OF CORTISONE BY LIVER HOMOGENATES. THE TPN SYSTEM WAS NOT ADDED

Group No.		No. of animals	Micrograms cortisone lost
1	Castrated males	6	45 ± 5
2	Estradiol treated males	6	19 ± 2
3	Castrated males treated with estradiol	6	16 ± 2
4	Ovariectomized females	7	25 ± 4
5	Testosterone treated females	6	30 ± 6

side chain reduction are presented in Table 2. The animals represented in this table were similar in age and weight to the normal rats of Table 1 and direct comparisons can be made. The operated male animals were castrated 15 days prior to removal of the liver for study. The females were ovariectomized 20 days before study. Gonadectomy is seen to have a marked effect on both the male and female but in opposite directions, i.e., gonadectomy decreases the rate of cortisone reduction in the male and increases the rate in the female. The male rats in Groups 2 and 3 of Table 2 were treated with estradiol-17β, 60 μg./day subcutaneously for 12 days. Cortisone metabolism is obviously depressed in these preparations.

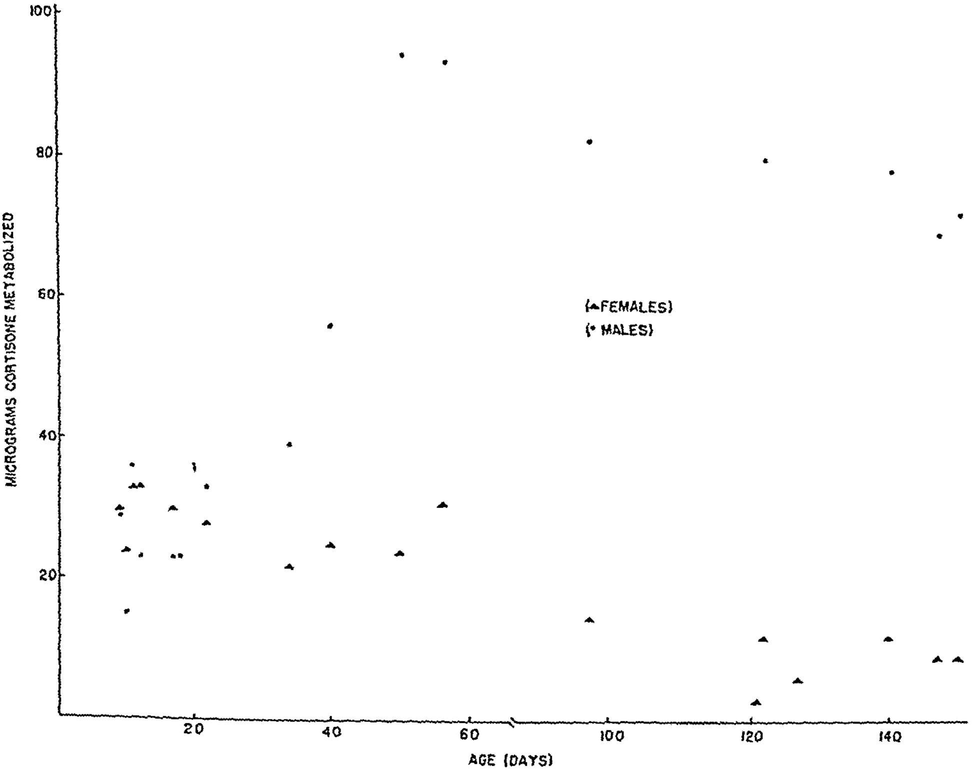

FIG. 1. Influence of age and sex on the metabolism of cortisone by liver homogenates. Cortisone was measured by the Porter-Siber reaction. The TPN system was not added.

Testosterone promotes the ability of liver tissue from female rats to reduce the sidechain of cortisone. This is shown in Group 5 of Table 2. These animals were given 2.5 mg. testosterone propionate per day for 15 days intramuscularly.

It was of interest to determine the age at which male and female rats first begin to metabolize cortisone differently. Figure 1 indicates no difference during the first 20 days of postnatal life. However, by 30 days of age the values for the two sexes are clearly divergent due to a sharp increase in the activity of male liver and a moderate decrease in that of female liver. The male appears to reach a peak value at about 50 to 60 days of age and to gradually decline thereafter.

DISCUSSION

The studies presented indicate that homogenates prepared from the liver of male and female rats metabolize the sidechain and A-ring of cortisone in a quantitatively different manner. The male reduces the sidechain more rapidly than does the female who, in turn, reduces the A-ring at a greater rate than does the male. This is in line with the observation of Yates and coworkers (9) that liver slices from female rats have a greater capacity for reducing the A-ring of cortisone than do slices from male rats. It is also of interest to note that the female rat metabolizes the A-ring to a greater extent than the sidechain (Table 1) while the male more nearly reduces these sites on the molecule to the same extent. This is especially the case in the presence of TPN. The ratio A-ring reduction/sidechain reduction for the female is 3.4; for the male it is 0.88.

The action of triphosphopyridine nucleotide in accelerating the rate of reduction of the sidechain and A-ring (Table 1) is in agreement with much previous work (4,6,7,8). Glenn and coworkers (13) working with the microsome-supernatant fraction of rat liver, have observed that the C-20 ketone group is metabolized faster than the Δ4–3 ketone function of hydrocortisone. However, alterations in substrate and cofactor composition were stated to be capable of reversing this process. Sex of the animals studied was not given and in view of the present findings this may be an important factor.

That the sex difference in the metabolism of the sidechain of cortisone is dependent upon gonadal secretions is clearly indicated by the data in Table 2, the male hormone tending to increase and the female hormone tending to decrease the rate of reduction. These effects are further pointed up in Figure 1 with the marked sex difference becoming apparent at about the time significant endocrine activity of the gonads would be expected.

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THE EFFECT OF ANTERIOR HYPOPHYSEAL ADMINISTRATION ON THE OVARIES OF OLD RATS[1]

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ABSTRACT

Following the removal of the left (control) ovaries, groups of immature, young adult and old rats received homologous anterior lobe implants daily for 10 days; the right (stimulated) ovaries were obtained at autopsy. The ovaries were examined in serial sections for the numbers and sizes of corpora lutea and follicles, and for the presence of "wheel cells" in the interstitial tissue.

The ovaries of the old rats showed a variable response (increase in weight and an increase in the number and size of follicles and of corpora lutea) to anterior lobe implants, but for the group as a whole, there were statistically no significant differences between the "control" and "stimulated" ovaries for the weights of the organs or the numbers of corpora lutea, and only borderline significance for the number of follicles. However, increase in the size of the follicles and corpora lutea in the stimulated ovaries was more pronounced and the differences, as measured by the mean diameter of these structures, showed a high degree of statistical significance. Wheel cells were present in 75% of the "control" ovaries and in only 29% of the "stimulated" ovaries. The reaction of the "stimulated" ovaries of the immature and young adult rats to anterior lobe administration was much more pronounced and relatively more constant than that which occurred in the old rats.

THE primary cause of ovarian regression which is associated with aging has not been definitely established, although waning anterior hypophyseal activity and a decreased capacity of the ovary to respond to stimulation by the anterior lobe have both been suggested. In old anestrous mice it has been found that a single implant of anterior lobe tissue induced an increase in weight of the ovaries accompanied by a growth of follicles, formation of corpora lutea and estrus (1, 2, 3). Following the administration of either anterior lobe tissue or urinary gonadotrophin, Weisner (4) noted essentially the same reaction in aged rats and expressed the view that the regression of the gonads was due to the lack of a specific stimulating factor. In contrast, in studies in which women of various ages received either pregnant mare serum (PMS) or an extract of castrate urine, Watson, Smith and Kurzrok (5) concluded that the causal factors in ovarian regression were inherent in the ovary itself and that this organ became refractive to gonadotrophic stimulation, a view also held by Masters (6).

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More recently Ortiz (7) found, correlated with increasing age, a small and gradual decrease of the response of the ovaries of hamsters to PMS, although the ovaries of old animals were still quite reactive. Finally, in mice ranging in age from 3 to 19 months, Green (8) reported that PMS induced a pronounced and fairly uniform increment in ovarian weight up to the age of 15 months; in increasingly old animals the response progressively decreased.

In studies (9, 10) pertinent to the present investigation it was found that, correlated with advancing age, the ovaries of rats were characterized by a progressive decrease in the number of normal follicles and corpora lutea, an increase in follicular atresia, and evidence of a partial failure of ovulation. In many animals, "wheel" or "deficiency" cells similar to those first described in the ovaries of hypophysectomized rats (11) appeared. Both a deficiency of anterior lobe stimulation and the incapacity of the ovary to respond to such stimulation have been suggested as causal in the appearance of these cells in old rats (9, 12). The present communication is concerned with the comparative responses of the ovaries of relatively old and relatively young female rats to anterior lobe administration.

MATERIALS AND METHODS

Female rats between the ages of 22 and 32 months and groups of immature (approximately 30 days old) and young adult rats (approximately 90 days old) received anterior lobe implants. Routinely, the left (control) ovary of each animal was removed; the animals then received 10 daily intramuscular implants of a fresh anterior lobe from a normal adult male rat. The animals were killed 48 hours after receiving the last implant, and the right (stimulated) ovary and the remainder of the reproductive tract were removed. All tissues were fixed in Bouin's fluid, embedded in paraffin, and stained with hematoxylin and eosin. Serial sections of the ovaries and representative sections of uteri and vaginae were studied.

The ovaries were examined by the quantitative methods described by Wolfe (9) The follicles and corpora lutea in each ovary were counted; their sizes were determined by taking the average of their two greatest diameters. Only follicles with a mean diameter of at least 0.3 mm. and corpora lutea with an average diameter of at least 0.5 mm. were included in the counts; follicles with an average diameter of 0.9 mm. or more were regarded as cystic.

In the ovaries of old rats, where relatively small numbers of follicles were present, the normal, atretic, and cystic follicles were counted separately; unless specifically noted, however, they will be considered below as a single group. In the younger rats, the stimulated ovaries contained so many follicles that it was impractical to count the three types separately. Since it was not feasible to measure the sizes of all the follicles and corpora lutea in the young animals, random sampling was used, in each case adequate in number to assure a statistically reliable average.

RESULTS

A statistical analysis of the over-all differences between the various responses to anterior lobe stimulation, such as weights, number and size of follicles and corpora lutea, of the control and stimulated ovaries of the old

TABLE 1. THE EFFECTS OF IMPLANTATION OF ANTERIOR LOBES ON OVARIES OF OLD RATS

Number of Rats = 28

	Control	Stimulated	Standard error (SD)	Mean 1–Mean 2
				SD
Mean weights of ovaries (mg.)	36 ± 4	44 ± 3	5.7	1.4
Mean number of follicles per ovary	9 ± .9	12 ± 1	1.4	2.1
Mean diameter of follicles (mm.)	.55 ± .03	.72 ± .01	.03	5.0
Ovaries with cystic follicles	43%	89%	—	—
Ovaries with corpora lutea	70%	89%	—	—
Mean number of corpora lutea per ovary	6 ± .7	8 ± 1	1.2	1.8
Mean diameter of corpora lutea (mm.)	.91 ± .05	1.13 ± .04	.05	4.4
Ovaries with direct luteinization	19%	56%	—	—
Ovaries with "wheel" cells	75%	29%	—	—

rats and of the younger animals is presented in Tables 1 and 2. The last column of these tables gives the significance ratio; a ratio between 2 and 2.5 is about 95% significant. Certain of these responses, or quantitative differences in the responses between the control and stimulated ovaries of individual rats, are indicated in Figures 1 and 2.

Reference to Table 1 will indicate that while in the old rats the mean weight of the stimulated ovaries was increased, the degree of the increase was not statistically significant. Furthermore, the responses among the individual rats were quite variable; in 19 rats the stimulated ovaries were heavier to varying degrees than the controls, but in 7 instances the stimulated ovaries actually weighed less than the controls, and in two rats there was no change (Fig. 1).

The mean number of follicles per ovary was slightly and barely significantly increased in the stimulated ovaries (Table 1), but again examination of data from individual rats indicates that the responses varied widely (Fig. 1). In direct contrast, the average size of the follicles was pronounc-

TABLE 2. THE EFFECTS OF IMPLANTATION OF ANTERIOR LOBE TISSUE ON THE OVARIES OF IMMATURE AND YOUNG ADULT RATS

	Immature rats No. = 9				Young adult rats No. = 8			
	Control Ovary	Stimulated Ovary	Standard error	M_1-M_2*	Control Ovary	Stimulated Ovary	SD*	M_1-M_2*
				SD				SD
Mean weights of ovaries (mg.)	14 ± .6	106 ± 14	14	6.5	49 ± 2	168 ± 24	24	5
Mean number of follicles per ovary	17 ± 1.7	52 ± 10	10	3.5	29 ± 3.9	71 ± 15.6	15.8	2.7
Mean diameter of follicles (mm.)	.39 ± .02	.60 ± .02	.02	10.5	.46 ± .03	.69 ± .05	.05	4.6
Ovaries with cystic follicles	0%	67%	—	—	0%	100%	—	—
Ovaries with corpora lutea	0%	100%	—	—	100%	100%	—	—
Mean number of corpora lutea per ovary	0	17 ± 2.8	—	—	15 ± 1.4	21 ± 3.2	4.2	1.4
Mean diameter of corpora lutea (mm.)	0	1.18 ± .06	—	—	.88 ± .04	1.26 ± .06	.07	5.4
Ovaries with direct luteinization	0%	67%	—	—	0%	50%	—	—

* M = mean. SD = Standard error.

edly increased after anterior lobe administration, the increase being highly significant (Table 1). Cystic follicles also occurred much more frequently in the stimulated ovaries (Table 1). However, the increased size of the follicles in the stimulated ovaries was not entirely due to the increased occurrence of cystic follicles, for when the normal follicles were considered alone, it was found that they were significantly increased in mean diameter.

The mean number of corpora lutea in the stimulated ovaries was slightly

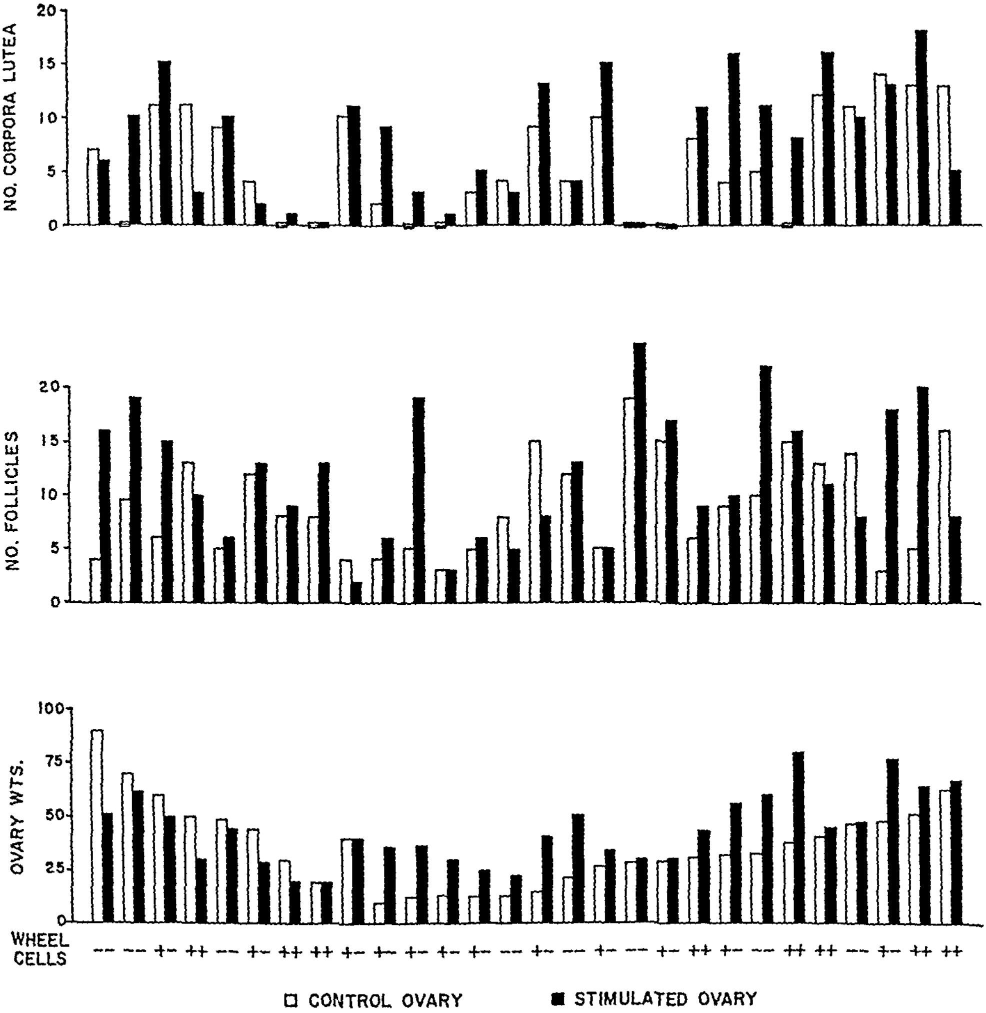

FIG. 1. The number of corpora lutea, the number of follicles and the weights, in mg., of the control and stimulated ovaries of the old rats are indicated by the three rows of bars which are arranged so that these three responses from a single animal are directly in line, one above the other. Control ovaries are represented by open bars, stimulated ovaries by solid bars. The very low bars cutting the base line in the first row indicate that no measurable corpora lutea were present. Also indicated at the bottom of the figure are the presence or absence of wheel cells in the individual ovaries.

greater than that in the controls, but the increase was statistically insignificant (Table 1), and it will be noted in Figure 1 that an increase actually occurred in only 16 rats; in the remainder there was either a decrease or no change. However, as in the case of the follicles, the corpora lutea were found in stimulated ovaries to be on the average significantly larger than those of the controls (Table 1). In many ovaries, histological examination revealed that numerous corpora lutea had formed by direct luteinization, as entrapped ova were found; the number of ovaries containing corpora lutea formed in this fashion was markedly increased in the stimulated ovaries (Table 1).

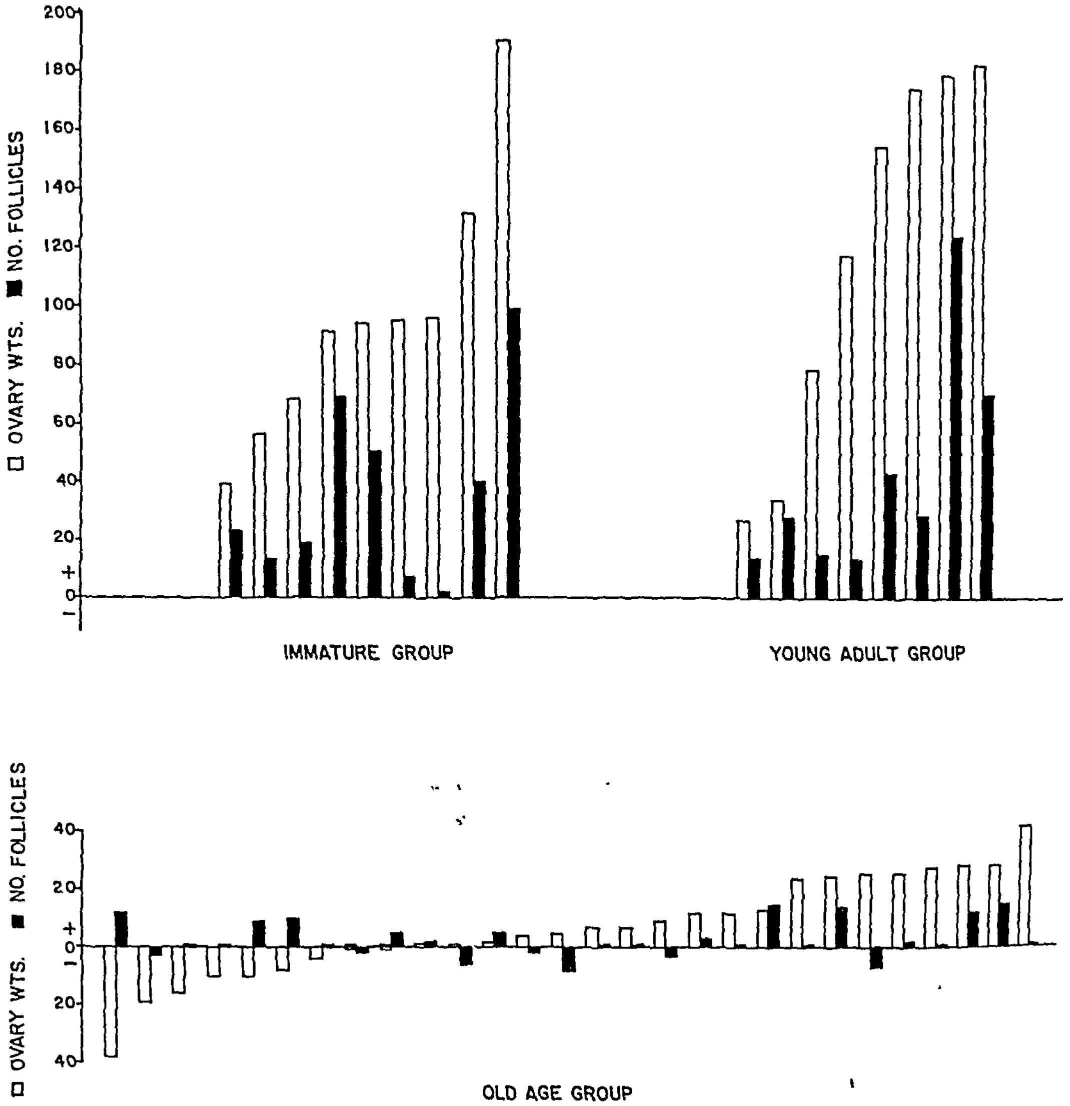

Fig. 2. The heights of the pairs of bars represent actual differences in the weights in milligrams (open bars) and in the number of follicles (solid bars) between the control and stimulated ovaries of individual rats. Negative differences (i.e., when a control ovary was heavier than the stimulated) lie below the base line, positive differences above. The very low bars cutting the base line indicate that no differences occurred.

Seventy-five per cent of the control ovaries contained wheel cells in the interstitial tissue, while such cells occurred in only 29% of the stimulated ovaries (Table 1). The absence or the presence of these cells in the control and stimulated ovaries of individual rats is indicated in Figure 1.

A statistical analysis of the over-all response of the ovaries of the immature and young adult rats to anterior lobe treatment is presented in Table 2. In general, the reaction was much more pronounced and much less variable than that in the old rats. The stimulated ovaries of both groups of the younger rats were significantly heavier than the control ovaries (Table 2). In Figure 2, where the difference between the weights of the control and stimulated ovaries of each rat is recorded, both the increased response of the younger rats and the lesser variability are quite clear.

The numbers of follicles were significantly increased in the stimulated ovaries of both groups of younger rats (Table 2). While the degree of such increase varied among the individual rats, the consistency of increased production of follicles in every ovary stimulated by anterior lobe tissue is evident from the fact that no columns are seen below the zero line (Fig. 2, upper row). Furthermore, the over-all response greatly exceeded that which occurred in the old animals, where the increase in the mean number of follicles was of only slight significance (Table 1) and actually occurred in only 19 of the 28 stimulated ovaries (Fig. 1).

The mean diameter of the follicles in the stimulated ovaries of both groups of young rats was significantly increased (Table 2), and there was a pronounced increase in the numbers of cystic follicles (Table 2), reactions which also occurred in the stimulated ovaries of the old rats.

In the young adult rats, it was found that the mean number of corpora lutea was not significantly increased after anterior lobe administration but that significant increase in size did occur (Table 2). In addition, increased numbers of luteinized follicles were found in the stimulated ovaries. In these responses, the old and the young adult animals responded similarly (compare Table 1 with Table 2).

The question arises whether there were consistent correlations between the various responses to anterior lobe administration in the individual rats. In both the immature and young adult rats such correlations were fairly consistent. As an example, it is shown in Figure 2 that the increase in weight of the individual stimulated ovary was invariably accompanied by an increase in the number of follicles in the same ovary. In the immature rats the increase in ovarian weight was accompanied by an increase in the number of corpora lutea; in the young adult rats, however, an increase in the number of corpora lutea failed to occur in three of the stimulated ovaries. In the ovaries of the old rats, there was no consistency among the various responses. An example of this finding is presented. Among the 19 old rats where the stimulated ovary was larger than the control, there was an increase in the number of follicles in 12 instances (Figs. 1, 2), disappearance of wheel cells in 9, and an increase in number of corpora lutea in 12 (Fig. 1). However, of the 9 rats in which the stimulated ovary either was no heavier or actually weighed less than the control, there was also an increase in the number of follicles in 7 rats (Figs. 1, 2), disappearance of wheel cells in 3, and an increase in the number of corpora lutea in 5 rats (Fig. 1).

In the above analysis the weight increase or lack of weight increase was used as a point of reference with which the other responses were compared. In a further analysis of the data presented in Figure 1, an increase or lack of increase in the number of follicles, an increase or lack of increase in the number of corpora lutea, and the disappearance or persistence of wheel cells in the stimulated ovaries were successively used as the base of reference. Again, a similar lack of consistency between the various responses was noted in all of these comparisons.

Testis-like tubules seemingly identical to those described by Engle and others (13, 14, 15, 16) were present in the ovaries of all of the old rats. Although not subjected to quantitative analysis, the tubules appeared to be equally abundant in the control and stimulated ovaries; thus, there was no evidence to indicate that they were affected by the anterior lobe administration. These tubules were not found in the immature animals, but a few occurred in the ovaries (both control and stimulated) of three of the young adult rats. The present findings, therefore, suggest that their appearance in the ovary of the rat is a manifestation of advancing age; this relationship is at present receiving further attention.

In the region of the hilum, the ovaries of 4 of the old rats contained large papillary intracystic proliferations resembling the papillary cystadenomata illustrated by Moon and his associates (15). Whether these are structural changes related to aging is open to further consideration.

DISCUSSION

The fact that the ovaries of most old rats are capable of responding to the administration of anterior lobe tissue would support the view that ovarian regression in aged rats is due to reduced activity of the anterior lobe. However, the lesser intensity of the response as compared to that found in younger rats, and the pronounced variability of the response, strongly suggest that the development of a refractory state in the ovary is an important factor in the decrease and in the final termination of gonadal activity in old female rats. The degree of response to stimulation shown by the ovaries of old rats varies within wide limits.

Unpublished studies which have been carried out in this laboratory have a bearing on this problem. Ovaries were exchanged between pairs of rats, one young and one old, by reciprocal transplantation into the kidneys. It was found, on the basis of observations on the estrous cycle and subsequent histologic examination of the transplant, that the transplantation of "young" ovaries in old rats was much more successful than the transplantation of "old" ovaries in young hosts. Thus, the factors determining the survival and function of the graft seemed to be predominantly inherent in the ovary itself. It should be noted, however, that Li and Gardner (17) have found that the ovaries of old mice developed tumors when transplanted into the spleen or the pancreas of young animals, and that they seemed to be just as capable of forming tumors under these conditions as did the ovaries of young mice. However, these two sets of experiments are not comparable, since the latter authors were determining the capacity of the ovary for atypical growth rather than its capacity for maintenance of normal structure and function.

Although the present studies strongly suggest that the ovaries of old rats become less responsive to stimulation by the anterior hypophysis, it is possible that lessened ovarian reactivity is not the only factor involved. Thus it has been reported (9) that correlated with advancing age in the female rat there is evidence of a decrease of secretory activity in the anterior lobe cells, and certainly, since ovarian and anterior lobe activity are so intimately linked, it is not unreasonable to assume that aging changes in the anterior lobe are concerned in ovarian regression. In her appraisal of this problem in hamsters, Ortiz (7) concludes that reproductive involution is not due to a complete loss of function on the part of the anterior lobe or to a refractive state of the ovary but rather to a decrease in the functional activities of many organs leading to an endocrine imbalance.

SUMMARY

The ovaries of aged, immature and young adult rats, before and after administration of homologous anterior lobe implants, were studied in serial sections.

The ovaries of the old rats responded to anterior lobe implants, but the degree of the response was reduced and the response was much more variable than that of the ovaries of younger rats.

The "stimulated" ovaries of the old rats were not significantly increased in weight, nor were the numbers of corpora lutea significantly increased. An increase in the numbers of follicles occurred, which was barely significant. In contrast, the mean size of the follicles was pronouncedly and significantly increased. The corpora lutea likewise were significantly increased in size. The ovaries of the immature and young adult rats responded more intensely to anterior lobe treatment. The increase which occurred in the weights of the ovaries and the increases in the number and size of the follicles were statistically significant. In the young adult group, the corpora lutea were significantly increased in size but not in number.

"Wheel" cells were present in 75% of the control ovaries of the old rats,

but were found in only 29% of the stimulated ovaries. Anterior lobe administration induced the formation of many cystic follicles and pronounced direct luteinization in the ovaries of both the young and old rats.

Comparison of the various categories of response by the stimulated ovary of each rat demonstrated a relatively consistent positive correlation in the immature and young adult rats, i.e., a positive response in one category was generally accompanied by other structural evidences of stimulation. Such positive correlations of responses were not evident in the old rats.

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THYROID HORMONE ADMINISTRATION AND OVARIAN AND ADRENAL ACTIVITY IN RATS BEARING HYPOTHALAMIC LESIONS[1]

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ABSTRACT

Bilaterally symmetrical electrolytic lesions which damaged arcuate nucleus and median eminence in the hypothalamus of adult female rats regularly produced atrophy of the reproductive tract (and reduced thyroid function) but were without consistent effect on adrenal morphology. Hypothalamic obesity and genital hypoplasia were frequently associated. Thyroxine and triiodothyronine administration to intact animals induced ovarian and uterine hypertrophy as well as adrenal enlargement. Similar treatment in operated rats failed to reverse the gonadal atrophy but did not interfere with the adrenal hypertrophy. A differential response was also found in rats made severely hypothyroid with propylthiouracil prior to hypothalamic destruction. Ovarian atrophy without concomitant change in adrenal cortex occurred following placing of lesions and goitrogen withdrawal. Evidences of adreno-cortical activation were seen in rats with diabetes insipidus (lesion-induced) and in chronically starved animals with marked hypothalamic deficit. The results signify that basal adenohypophysial ACTH secretion can be enhanced by chronic stress despite arcuate-median eminence lesions and that some aspects of thyro-ovarian interaction may be mediated through a hypothalamic-hypophysial component.

A FUNDAMENTAL role of the hypothalamus in the regulation of pituitary trophic hormone function has been securely established although pressing questions remain unanswered (1,2). The physiological anatomy underlying hypothalamic-hypophysial mechanisms is still obscure and the degree of anterior pituitary hormone secretion which escapes a central mechanism has not been precisely assessed. There is much evidence which suggests that in the rodent the dependency of pituitary-thyroid, adrenal, and gonadal systems on the hypothalamus differs markedly. A succession of recent experiments on the rat has brought into clearer focus the role of the hypothalamus in pituitary-thyroid interplay. It has been shown that anterior hypothalamic lesions selectively interfere with adenohypophysial TSH secretion under either augmented (3–9) or normal demand conditions (8, 10), and that as a consequence thyroid function is reduced. The residuum

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of TSH function in the adenohypophysis after hypothalamic deficit is nevertheless appreciable and the pituitary-thyroid servomechanism remains operative (11, 12). The disturbances which ensue in the genital system after hypothalamic damage are marked; loss of reproductive rhythm with or without gonadal atrophy is frequently observed (13–23). Differential control of the various pituitary gonadotrophins by the hypothalamus is still a matter of conjecture however. In contrast to the gonad, the morphologic consequences to the adrenal from destruction of the hypothalamus are relatively mild (24) although functional impairment of the adrenal cortex is known to exist (25–28). The relative importance of the hypothalamus in ACTH regulation under circumstances of acute versus chronic stress has not been accurately defined. Another unresolved point concerns the locus of adreno-cortical steroid inhibition in the feedback mechanism.

In view of the established importance of the thyroid gland in facilitating certain phases of reproductive activity (29, 30) and influencing adreno-cortical physiology (31), the possibility cannot be discounted that some of the histo-physiologic changes in ovary and adrenal of animals with hypothalamic lesions are non-specific consequences of lowered thyroid hormone levels. In a previous study (11) the effect of exogenous thyroid hormone on the hypothalamic-hypophysial-thyroid system of lesioned rats was analyzed in detail, with incidental observations made also on the other endocrine glands. The present investigation is an extension of this basic study and will be concerned primarily with the response of the adrenal and ovary to experimental manipulation of endogenous and exogenous thyroid hormone levels in the operated rat.

MATERIALS AND METHODS

The bilaterally symmetrical lesions were produced in the hypothalami of adult female albino rats by conventional stereotaxic methods (11, 12). The tip of the coagulating electrode was placed 6 mm. anterior to the external auditory meatus and .5 mm. on either side of the sagittal suture, with electrode placement varied in the vertical dimension (.5–1.5 mm. from skull base). A direct current of 2 milliamperes for 20 seconds duration was used. All rats received tap water and a ration of Purina dog checkers *ad libitum*. Operated rats were allowed to recover for at least 10 days before utilized in further experiments. Lesion localization was determined microscopically on Weil and toluidine blue stained coronal sections (12 micra) through the diencephalic area of formalin fixed brains. At various time intervals postoperatively, water intake and urine output were measured over a 72-hour period in rats maintained individually in special metabolic cages. The effects of varying the thyroid hormone level on the endocrine system of operated rats was tested in two major experiments. In the first, rats were treated postoperatively with various dose levels of sodium-*l* triiodothyronine (.4, 2 and 20 μg.) and sodium-*l* thyroxine (10 μg.) made up in 0.9% NaCl (pH. 8.5). The hormones were administered in constant volume (.5 ml.) by daily subcutaneous injections throughout the last two weeks of the post-lesioning period (24–70 days). In the second experiment, rats were made severely hypothyroid prior to electrocautery by feeding propylthiouracil in the ration (.1%) for 30 days. Goitrous rats were lesioned and immediately returned to the normal diet until sacrifice (11–21 days). The goitrogen was similarly withdrawn from unoperated animals in parallel experiments. At autopsy, the hypophysis, thryoid, adrenal and portions of the reproductive tract were quickly dissected, weighed on a micro-torsion balance, and then placed in suitable fixatives in preparation for histological study. The finer structure of the adenohypophysis was examined on Helly fixed, paraffin embedded serial sections cut at thicknesses of 3 micra through the horizontal plane. Masson staining was used routinely with histochemical stains applied to alternate tissue sections for identification of glyco-protein granulation in the basophils (11, 12). The other endocrine tissues were studied after fixation in Bouin's picro-formol solution and staining with Harris' hematoxylin and eosin or with the Masson stain.

RESULTS

Localization of the lesion

Although there were differences in the site and extent of necrosis, the center of the lesion was usually at the juncture of the anterior and middle thirds of the ventral hypothalamus. Typically, both areas of electrolysis were confluent in the mid-line (Fig. 1). The inclusive area of coagulation extended rostrocaudally from the region of the paraventricular nucleus to the mamillary body (Fig. 2). The degree of damage at rostral and caudal extremes was relatively slight and difficult to assess. In most instances, suprachiasmatic and paraventricular nuclei were spared but ventricular

All figures (except 2 and 10) are photo-micrographs taken with "B" and "G" filters

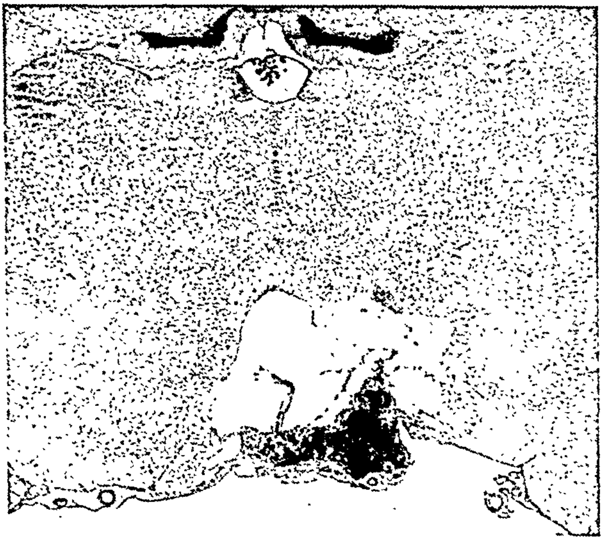

FIG. 1. A coronal section through the diencephalon of an obese (476 gm.) lesioned (56 days) rat which received thyroxine. The floor of the hypothalamus is well vascularized and intact but arcuate nucleus and median eminence are considerably damaged. Thyroid hormone failed to correct the severe ovarian atrophy (paired ovaries, 44 mg.) but induced adrenal hypertrophy (toluidine blue, ×15).

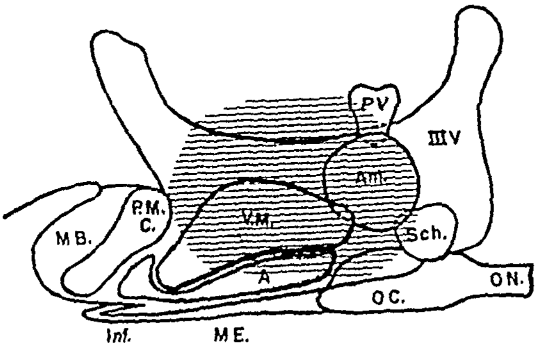

Fig. 2. Diagrammatic representation of the rat's hypothalamus (modified after Krieg, 46; Greer, 2) depicting the major nuclei damaged by electrocautery. Dorsally placed lesions (horizontal hatched area) affected thyroid function without apparent effect on the ovary; both thyroid and ovary were affected with lesions in the ventral hypothalamus (stippled area) IIIV. = third ventricle; O.N. = optic nerve; O.C. = optic chiasm; Sch. = suprachiasmatic nucleus; Ant. = anterior hypothalamic nucleus; P.V. = paraventricular nucleus; V.M. = ventromedial nucleus; A. = arcuate nucleus; M.E. = median eminence; P.M.C. = premamillary nuclear complex; M.B. = mamillary body; Inf. = infundibulum.

(3rd) distension in this region was commonplace (Fig. 3). Damage to the posterior hypothalamus was slight. Some destruction of neighboring fiber tracts (medial forebrain bundle, fornix, mamillo-thalamic) inevitably occurred and relationships of the 3rd ventricle were always disturbed. Lesions deeply placed in the ventral hypothalamus destroyed the periventricular grey area and median eminence, as well as damaging to some extent anterior hypothalamic and dorso- and ventro-medial nuclei. Such lesions produced atrophy of the reproductive tract and disturbances in the pituitary-thyroid system. Lesions more dorsally placed which destroyed dorso- and ventro-medial and anterior hypothalamic nuclei but spared the arcuate nucleus and median eminence affected the pituitary-thyroid system without apparent effect on the ovary. The degree of impairment in thyroid function with either type of lesion was not greatly different (12).

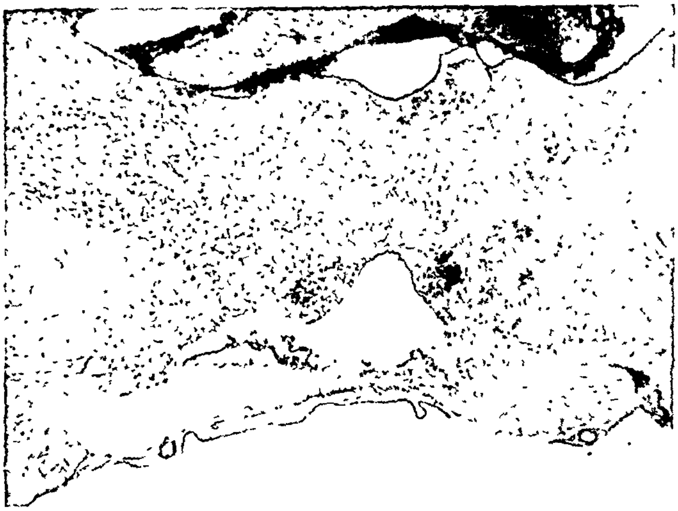

Fig. 3. The degree of damage in the anterior hypothalamus of the rat described in Figure 1. There is ventricular distension and some condensation of cells in the paraventricular nuclei; damage is otherwise relatively slight (toluidine blue, ×15).

TABLE 1. EFFECT OF VARYING THYROID HORMONE LEVEL ON ADRENAL AND OVARIAN ACTIVITY IN RATS BEARING HYPOTHALAMIC LESIONS

Group	No of rats	Mean body weight (gm) At operation	Mean body weight (gm) At initiation of hormone treatment	Mean body weight (gm) At sacrifice	Days lesioned	Mean gland weight (mg) Adrenal	Mean gland weight (mg) Level of significance[2]	Mean gland weight (mg) Ovary	Mean gland weight (mg) Level of significance[2]
Expt 1									
Normal Control Weight	12			386±32[1]	0	59 4±3 5	Pn s	103 4±9 3	P< 001
Lesion, Untreated Obese	6	273± 7		453±29	60–67	70 4±7 2		57 8±6 4	
Lesion, Untreated Non-obese	6	271±13		318±44	60–67	55 3±5 9	Pn s	69 6±7 4	Pn.s
Normal "Age" Control	18			285±40	0	60 6±3 2		74 8±3 4	
Lesion + 10 µg T Obese	8	237± 6	355±32	390±43	48–64	63 7±1 7	P< 005	53 9±5 6	P< 001
Lesion + 10 µg T Non-obese	13	246±14	268±34	276±47	48–64	53 2±2 3	P< 001	54 5±5 9	
Intact+10 µg T_4	15		308±25	305±23	0	69 8±3 1		93 4±4 9	
Expt 2									
On PTU, 30 days	11			271±35	0	44 0±3 4	Pn s	66 8±4 7	P< 05
Lesion, off PTU	18	269±30		240±27	11–21	47 7±2 3		53 1±5 4	P< 01
Intact, off PTU	18	268±46		275±47	0	48 2±1 9		72 6±5 5	

[1] Mean body weight ± average deviation, in other columns (gland weight) ± values refer to the standard error of the mean

[2] Statistical significance calculated from "T" test tables "P" values > 05 are not considered significant (n s)

Morphologic Effects of Lesion

The morphologic response of the ovary is statistically evaluated in Tables 1 and 2. Ovarian atrophy was found in both obese and non-obese operated rats but was more severe and occurred more often in the former. The genital hypoplasia was grossly visible in rats with hypothalamic obesity; ovaries were yellow and fibrous in appearance and uteri pale and thread-like. A suggestion as to the efficacy of the lesion was evident from gross inspection of the floor of the hypothalamus at autopsy. With severe atrophy of the reproductive tract, the ventral hypothalamus rostral to separation of the hypophysial stalk from median eminence presented an irregular rather than smooth surface and displayed brown discoloration. The marked regression of the gonad in obese rats became more evident when comparison was made with older intact rats of approximately the same weight ("weight controls") rather than with animals of similar age

TABLE 2. DIFFERENTIAL RESPONSE OF ADRENAL AND OVARY TO TRIIODOTHYRONINE IN RATS BEARING LESIONS

Treatment	No of animals	Mean body weight (gm) Initiation of hormone treatment	Mean body weight (gm) At sacrifice	Adrenal	Mean organ weight (mg) 100 gm final body weight % change	Mean organ weight (mg) 100 gm final body weight Ovary	% change
Intact—Saline	16	240±31	257±30[1]	22 5± 7		25 9±1 1	
Intact— 4 µg T_3	14	290±33	287±22	22 9± 7	0	27 0±1 3	+ 4 2
Intact—2µg T_3	18	234±21	249±18	26 7± 9	+18 7*	36 5±1 5	+40 9*
Intact—20 µg T_3	15	242±31	238±22	33 0±1 2	+46 6*	38 8±1 7	+49 9*
Lesion—Saline	14	215±29	238±36	21 0±1 1		20 4±1 4	
Lesion— 4 µg T_3	10	296±24	334±38[2]	17 2± 9	−18 1*	19 8±1 4	− 2 9
Lesion—2 µg.T_3	13	270±34	273±27	27 6±1 4	+31 4*	23 4±1 7	+14 7
Lesion—20 µg T_3	11	288±34	249±36	36 7±2 6	+74 6*	23 5±2 8	+15 2

[1] Mean final body weight ± average deviation, in other columns ± values represent standard errors of the mean.

[2] Some animals in this group were obese

* Mean percent changes (from appropriate saline control value) which are highly significant statistically (P" <.005) Other values are not deemed significant ("P" > 05).

("age controls"). Calculation of mean ovarian-body weight ratios in markedly obese animals yielded values less than 50% of normal; in some, ovarian atrophy approached the degree found after hypophysectomy. Destruction of the arcuate nucleus appeared to be a necessary prerequisite for reduction in weight of the ovary. Damage to the median eminence was an invariable accompaniment but some gonadal atrophy was found with either rostral or caudal portions of this neural structure apparently undamaged.[2]

The histologic changes in the gonad were clearly separable into two major categories. With marked atrophy, the structure of the ovary was preponderantly follicular. Small, medium and large follicles measuring between 500–700 micra in diameter, were present in healthy condition (Fig. 4). There was much atresia, and interstitial gland material, deeply basophilic in staining reaction, was abundant. Wheel-like nuclei were commonplace and deposits of yellow pigment were substantial. Corpus luteum formation was inhibited. When present, corpora lutea were small (less than 500 micra in diameter) and undergoing luteolysis. The second category of histologic alteration from normal was usually found in ovaries of nonobese lesioned rats in which only moderate atrophy occurred. These were characterized by the presence of well developed, histologically active corpora lutea, some over 1 mm. in diameter. In contrast, the follicular apparatus appeared suppressed. Few follicles had developed beyond the early antrum stage and none greater than 200 micra in diameter was visible. Regardless of the histologic picture in the ovary, the uterus was small, avascular and bore a slit-like lumen. The endometrium was either in the diestrous condition or similar to that in the spayed rat (Fig. 5). The luminal epithelium was smooth and of the cuboidal or low columnar type. No mitoses were visible. The uterine glands were collapsed and stained deeply, and fibro-muscular tissue was condensed.

Adrenal morphology was not consistently affected by hypothalamic

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[2] The degree of vascular disturbance engendered in the adenohypophysis after experimental manipulation either at hypothalamic or pituitary stalk levels bears importantly on the question of neural regulation of pituitary trophic hormone function (1, 45). In the present experiments, encroachment of some lesions on the primary capillary network in the median eminence was inevitable. The extent of damage at this site was not quantitated by special methods. Several sets of facts subvert the probability that variable damage to this component of the hypophysial portal system was primarily responsible for the major endocrinologic changes which ensued after operation. First, appreciable damage to the primary plexus might be expected to alter substantially the vascular anatomy and histologic integrity of the anterior pituitary; instead, the pars distalis appeared healthy and displayed minimal vascular change with little or no infarction. Secondly, the degree of impairment of the pituitary-thyroid system found after median eminence lesions was little greater than that which ensued when this area was spared. Finally, the post-operative period was of sufficiently long duration to expect that portions of the hypophysial portal system temporarily damaged would have regenerated.

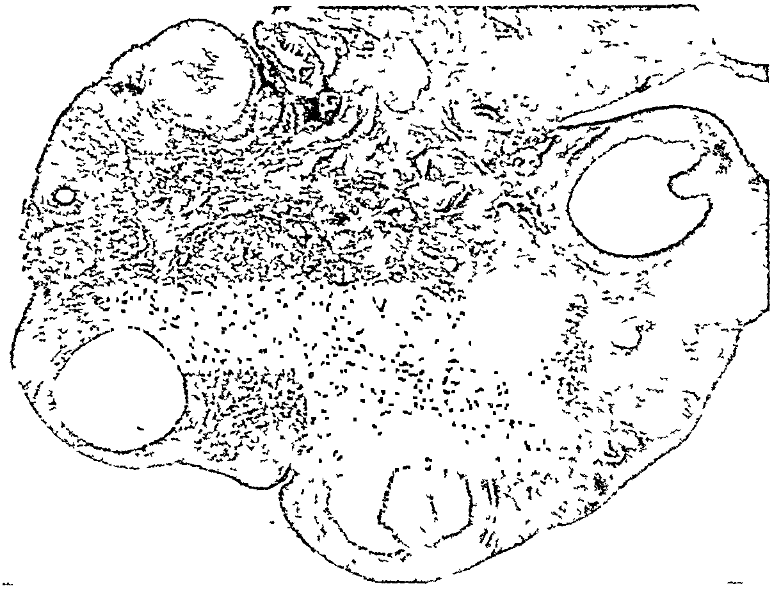

FIG. 4. Follicular character of the atrophic ovary in the untreated rat with lesion (70 days); the interstitium is well developed but no corpora lutea are present (hematoxylin and eosin, ×35).

destruction (Tables 1 and 2). Absolute weight of the adrenal in obese rats could be greater than that in either age or weight controls, with adrenal-body weight ratios not significantly altered. The histologic appearance of adrenal cortex and medulla in the operated rat was variable but no more so

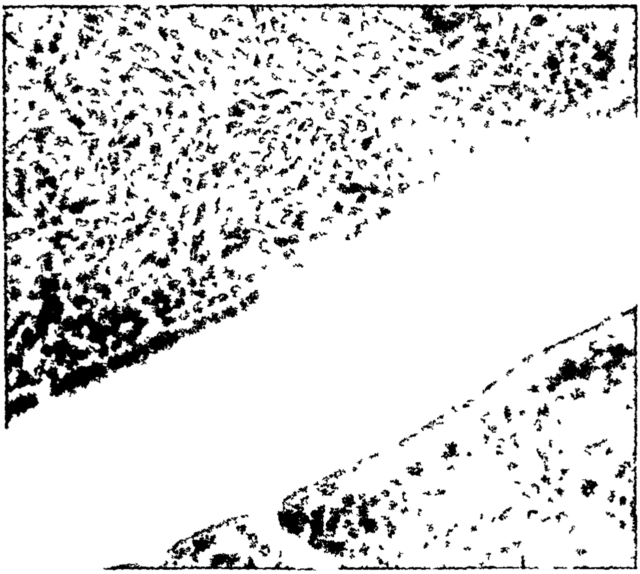

FIG 5. Typical histologic appearance of the endometrium in the rat described in Figure 4 The regression in epithelium and stroma approximates that found in the spayed animal (hematoxylin and eosin, ×400).

than in intact controls. Several isolated instances of adrenal atrophy did occur but these were associated with adenohypophysial rather than hypothalamic damage.

Effects of varying thyroid hormone level

Thyroid hormone administration produced marked stimulation of the reproductive tract in the unoperated rat. The ovaries were very vascular and enlarged and the uteri grossly hyperemic and swollen (Tables 1 and 2). The ovaries were heavily luteinized, with fresh corpora lutea and those of older orders visible (Fig. 6). Some of the larger corpora lutea were over 1.2 mm. in diameter and appeared histologically active. Healthy follicles in all stages of development were present. The uterine and vaginal histological structure closely resembled that seen with continued estrogen stimulation. The tall luminal epithelium in the endometrium contained many mitoses (Fig. 7). The enlarged uterine glands displayed proliferative changes and penetrated deeply into the stroma. There was extensive leucocytic infiltration of stroma and muscles layers. The latter were thickened and extended into the mesometrium. The structural changes in the vagina were indicative of sustained activation. The stratified epithelium was thickened and cornified with numerous mitoses present in the basal layers (Fig. 8). There was appreciable desquamation of the superficial cornified elements.

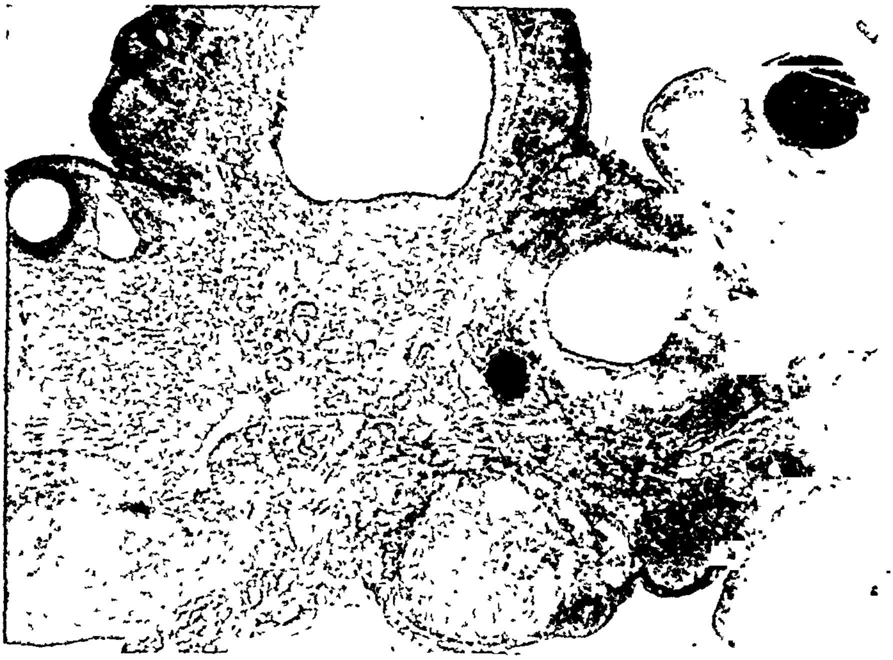

FIG. 6. The morphologic response of the ovary to thyroid hormone administration in the intact rat. The enlarged ovary contains many large follicles and well developed corpora lutea (hematoxylin and eosin, ×35).

The histology of the reproductive tract in lesioned rats receiving thy-

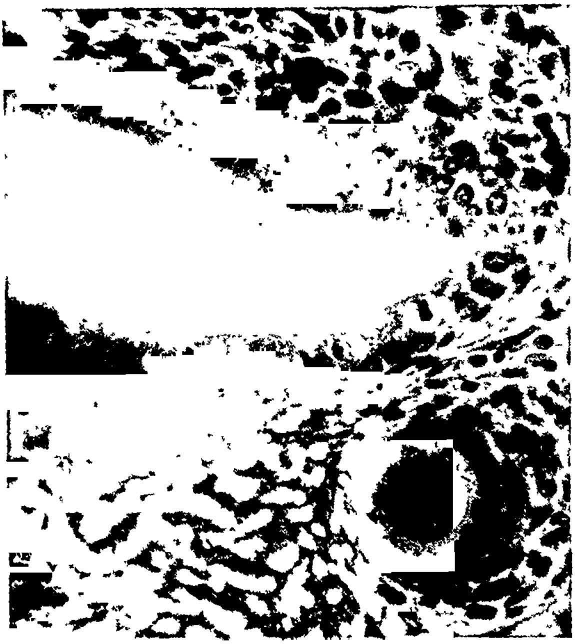

FIG. 7. Uterine hyperplasia which accompanied ovarian stimulation with exogenous thyroid hormone; numerous mitoses are visible in the hypertrophic epithelium (hematoxylin and eosin, ×400).

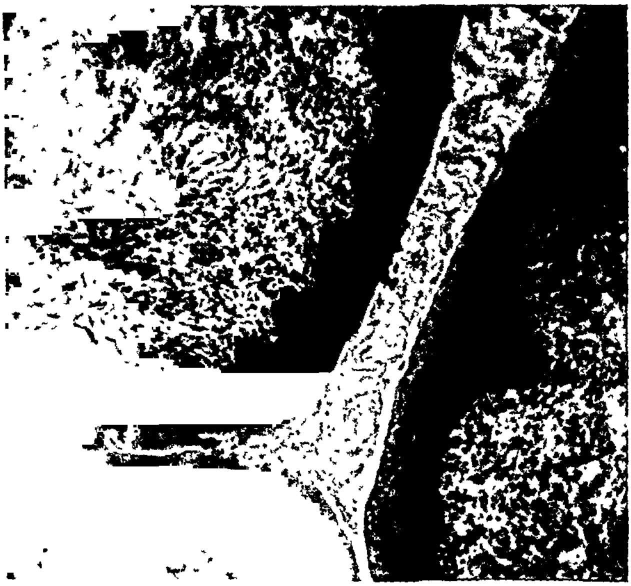

FIG. 8. The vaginal structure corresponding to the gonadal and uterine changes shown in Figure 6 and 7. The thickened epithelium is cornified, with much desquamation of superficial elements into the lumen (hematoxylin and eosin, ×100).

roid hormone contrasted highly with that in the intact animal. The ovarian atrophy was not corrected (Table 1 and 2) and the histology of the gonad appeared essentially unchanged (Fig. 9). The small uteri contained slit-like lumina lined by low columnar or cuboidal epithelium. No glandular activity was evident and the fibro-muscular tissue was condensed. The vaginal epithelium was thin with superficial layers consisting of mucified columnar cells. Luminal concentration of leucocytes was marked.

In contrast to the differential effects on the gonads, exogenous thyroid hormone induced substantial adrenal hypertrophy in lesioned rats which paralleled that found in intact controls. Triiodothyronine at higher dose levels was a potent stimulus to adrenal enlargement while inhibiting body weight gain. The 10 μg. dose of thyroxine used was less effective in causing adrenal hypertrophy. It retarded but did not abolish the weight gain in obese animals. The lowest dose of triiodothyronine used (.4 μg.) exerted no significant influence on adrenal weight, nor did it interfere with the development of hypothalamic obesity. The histology of the enlarged adrenal revealed activational changes restricted largely to the fasciculate zone of the cortex with the medulla relatively little affected.

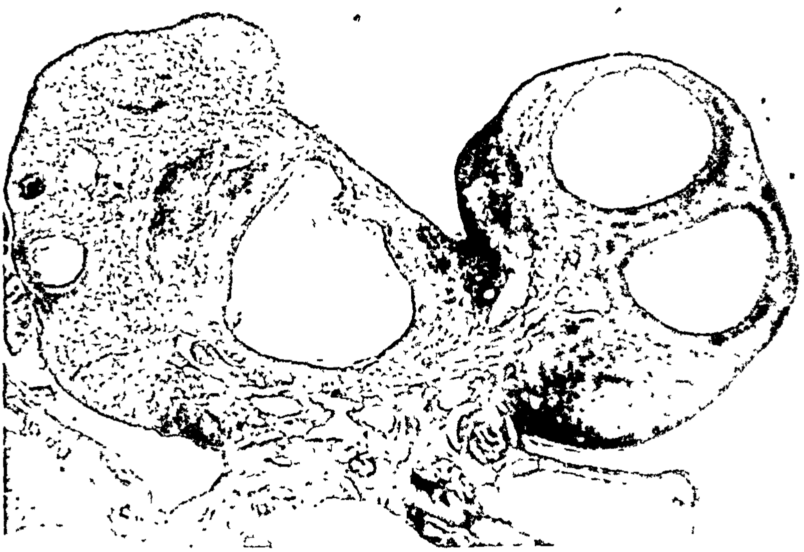

FIG. 9. Lack of effect of thyroid hormone on the gonad of a rat with hypothalamic lesion. The histologic picture of the atrophic ovary remains predominantly follicular (hematoxylin and eosin, ×35).

The morphologic response of adrenal and ovary in rats made severely hypothyroid with propylthiouracil prior to hypothalamic lesioning also differed significantly when the goitrogen was withdrawn (Table 1, Experiment 2). Pretreatment with the goitrogen for one month resulted in the anticipated thyroid hyperplasia (3–4 fold) and adrenal atrophy. There was no significant change in weight or histology of the ovary during this regimen. In both operated and intact rats adrenal gland weight was still subnormal 2–3 weeks after propylthiouracil withdrawal but evidences of ovarian atrophy were present only in the lesioned group.

A cyto-physiologic and histochemical analysis of the adenohypophyses of most lesioned rats used in this study has been considered in detail elsewhere (11, 12). The additional information derived from this investigation closely conforms. In brief, pituitary infarction was infrequent and aside from sinusoidal distension the vascular architecture was little affected. There was condensation of all cellular types in the parenchyma with many acidophils in advanced stages of degranulation. Significant shifts in the numbers and cytologic activity of beta and delta basophils occurred which correlated well with lesion placement and gonad and thyroid function. The delta basophils responded more sensitively to hypothalamic destruction, displaying marked change in rats with ovarian atrophy. Characteristic clustering of these gonadotrophes along the portal vessels was lost and their glyco-protein granulation was distinctly reduced. Despite the most extensive lesions, the beta basophils (thyrotropes) persisted and these retained their elective affinity for aldehyde fuchsin. Thyroxine and triiodothyronine induced drastic changes in the chromophilic cell types of the adenohypophyses in operated rats. Regranulation and staining intensity of acidophils were increased. Beta cells almost completely disappeared. There was a return of delta basophils to their customary locations in dorsal and ventral regions of the gland. The alterations in the basophilic cell types in thyroid treated lesioned rats appeared qualitatively similar to those in intact animals. (The quantitative implications will be described in another study.)

Effects of diabetes insipidus and starvation

In table 3 are indicated the adrenal gland weights of individual lesioned rats in which water intake and urine output were measured just prior to thyroxine treatment. Group A is comprised of animals in which diabetes insipidus ensued as a consequence of hypothalamic destruction (rats with urine volumes at least twice greater than the mean of unoperated rats were arbitrarily considered to be polyuric).[3] Inspection of adrenal weight values in polyuric (Group A) and non-polyuric lesioned rats (Group B) receiving identical thyroxine treatment reveals that in the former the adrenal is not only well maintained but also has the capacity to enlarge despite damage to the neuro-hypophysis. Adrenal hypertrophy in the lesioned rat was not

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[3] In view of the fact that in hypothalamic obesity the metabolic mass is not the same as that of intact rats at similar body weight, some reservation must be held regarding expression of urine output in terms of "cc./hours/body weight." There is no doubt however that a true diabetes insipidus existed and actually may have been more marked than the values indicate.

TABLE 3. ADRENAL WEIGHT RESPONSE TO EXOGENOUS THYROID HORMONE IN LESION-INDUCED POLYURIC RATS

Group	Final body weight (gm.)	Days lesioned	Urinary output cc../100 gm. body weight/24 hr.	Adrenal weight (mg.)
A. Lesioned, Polyuric	332	50	57.0	49.4
	360	51	11.4	71.0
	454	51	10.6	67.5
	404	57	12.5	60.0
	384	58	14.9	56.5
	425	120	8.5	81.4
	400	120	14.5	93.0
	284	120	34.5	65.0
Mean	381		20.5	68.0
B. Lesioned, No Polyuria	250	50	5.5	50.4
	234	51	3.0	57.2
	302	57	5.0	52.0
	266	58	2.7	61.0
	218	210	1.9	44.5
Mean	254		3.6	53.0
C. Mean of 18 Intact "Age" Controls	290	0	3.9	60.6
D. Mean of 11 Intact "Weight" Controls	391	0	3.3	64.8

Urine output was measured just prior to initiation of thyroxine treatment (10 µg. daily, for last two weeks of experiment).

restricted to experimentally induced hyperthyroidism. It occurred in animals which ate little or no food after operation or in those from which food and water were withheld by experimental design. In a group of 14 starved rats sacrificed 6–25 days after electrocautery (28.5% mean body weight loss) all but one responded with either relative or absolute increase in weight of the adrenal (Fig. 10). In 7 of these rats, adrenal weight was greater than it would be prior to starvation.

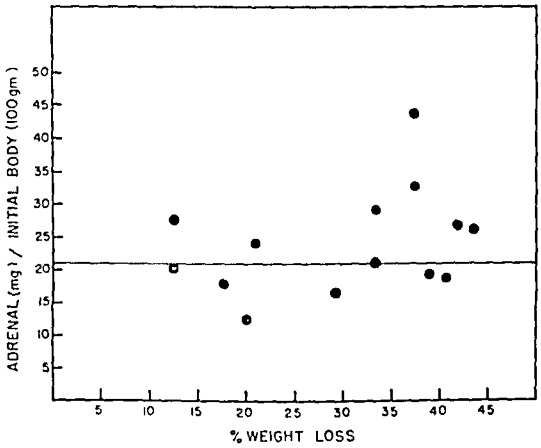

FIG. 10. Effect of starvation on adrenal hypertrophy in lesioned rats. The horizontal line represents the mean adrenal-body weight ratio in normally fed rats (225–275 gm. body weight). Values for individual operated rats (circles) subjected to chronic starvation are related to the pre-starvation rather than final body weight. The adrenal hypertrophy is plotted as a function of body weight loss (%) regardless of the number of days the rat was lesioned.

DISCUSSION

The association of gonadal atrophy and hypothalamic obesity has been noted often; the relationship is not causal and different mechanisms are involved (32, 33). In this study, the genital hypoplasia was clearly referable to secretory alterations in the adenohypophysis which were not contingent on the presence or absence of obesity. Similar interpretation was made by Bogdanove and associates (6, 7) and Van Dyke *et al.* (23) in the male and female rat respectively. The severity of the ovarian atrophy which followed destruction of the periventricular grey area and the median eminence pointed to these neural structures as being of crucial importance in the hypothalamic-hypophysial pathway governing structural and functional integrity of the ovary. That varying degrees of gonadal atrophy, with or without disturbances in reproductive rhythm, result from generalized damage to the tuberal area in the rat has been reported (16, 17, 23, 34) but the relationship of this hypothalamic region to a more rostral one controlling the sex cycle is rather obscure. With anterior hypothalamic lesions, disturbances in cyclicity without gonadal atrophy occur. It was found in both guinea pig (13–15) and rat (18–20) that destruction of the area from optic chiasm to rostral edge of the median eminence produced persistent estrus, hypertrophied uteri and markedly follicular ovaries in which corpus luteum formation was inhibited; gonad weight was unchanged.

The hormonal alterations responsible for the imbalance in the pituitary-ovarian system after hypothalamic damage have not been elucidated. Dey (15) and Hillarp (18) postulated that anterior hypothalamic lesions curtailed LH secretion while sparing the FSH mechanism in the adenohypophysis, thus permitting continued estrogen secretion from the follicular ovaries. The induction of corpus luteum formation in such ovaries with luteinizing hormone preparations (23, 35) reinforces the view that pituitary LH secretion is deficient in the lesioned animal but selective failure in LH function cannot explain some of the reproductive phenomena which result. Greer (19) was able to re-establish normal cycles and corpus luteum formation with progesterone administration in persistent estrus rats bearing anterior hypothalamic lesions; pseudopregnancy was also produced upon cervical stimulation. The closest counterpart to this in the intact animal was described by Everett (36) in a spontaneous estrus strain in which correctly timed injections of sub-inhibitory quantities of progesterone restored regular ovulatory cycles. If the possibility of hypothalamic re-organization in Greer's experiments is discounted, it would appear that balanced gonadotrophic hormone secretion by the pituitary can be restored by progesterone in the absence of the hypothalamic stimulus and despite the fact that some effects of this ovarian hormone are known to be mediated through the central nervous system. The crucial element in the hypothalamic-hypophysial-gonadal interplay may involve primarily an estrogen-FSH mechanism with changes in LH secretion secondary. In a series of trenchant observations, Flerko (20, 21, 22, 47) demonstrated that a region in the hypothalamus just ventral to the paraventricular nucleus is sensitive to changing levels in circulating estrogens and constitutes an essential link in in the reciprocal interaction between ovary and adenohypophysis. In rats with this area destroyed much more estrogen than normal was required to inhibit pituitary FSH secretion. Conversely, ovarian autografts in the same region resulted in reduced uterine weight and diestrus, responses indicative of FSH *suppression. Grafts at other sites (posterior hypothalamus,* hypophysis and liver) were ineffective. The results were considered to disprove the concept that estrogen inhibits FSH secretion directly at the adenohypophysis. It must be emphasized that although the effects of ovarian hormones on gonadotrophic hormone functions are mediated in part through neural mechanisms, direct action at pituitary levels is not necessarily excluded. Rose and Nelson (37) evoked substantial cytologic change in the pars distalis of rats with local administration of estradiol into the hypophysial fossa, a finding consistent with the classic interpretation of a pituitary-ovarian feedback mechanism.

The best interpretation that can be made from the present results is that destruction of the arcuate nucleus-median eminence complex seriously interfered with both FSH and LH secretion by the anterior pituitary. Although vaginal smears were not made in lesioned rats, the histologic picture of the reproductive tract was consistently one of atrophy. An absolute deficiency of estrogen secretion must have occurred in all atrophic ovaries irrespective of corpus luteum inhibition or apparent sparing of the follicular apparatus. The histochemical changes in the adenohypophysis conformed closely. The decreased size and number of growing follicles and the abundance of wheel-cell formation in the interstitial tissue of severely atrophic ovaries correlated strongly with drastic reductions in both central and peripheral gonadotrophs in the pars distalis. The totality of histophysiologic change in the pituitary-ovarian system resulting from arcuate nucleus-median eminence lesions simulated that which ensues from pituitary transplantation or severance of its stalk. Whether or not luteotrophin continues to be secreted after the placement of lesions which curtail FSH and LH secretion remains to be tested. There is impressive evidence that vigorous and prolonged luteotrophin secretion does not require an intact hypothalamic connection, even though some modulation of hormone secretion by the hypothalamus is quite probable. Luteotrophin is secreted from rat pituitaries transplanted to the kidneys very likely instead of other gonadotrophins, and in amounts sufficient to maintain functional corpora lutea for months (38).

The manner in which the anterior and tuberal hypothalamic "centers" of the rat are integrated to achieve balanced gonadotrophic hormone secretion from the anterior hypophysis defies analysis at present. Without direct measurement of the levels of the individual gonadotrophins in blood and pituitary of lesioned rats, differential regulation of FSH and LH secretion by either hypothalamic zone cannot be assessed. It would be expected that stimuli from the rostral hypothalamus—whatever their nature—should inevitably converge in or near the median eminence region as a focal point in the hypothalamic-hypophysial pathway for gonadotrophic hormone regulation. Destruction of median eminence and associated periventricular grey would therefore eliminate all hypothalamic influence regardless of its source and should lead to the greatest gonadotrophin deficit and gonadal atrophy. It is significant that in the male rat severe gonadal atrophy also occurs after destruction of the arcuate nucleus-median eminence complex (7, 25) and that under these circumstances pituitary gonadotrophin is drastically reduced (7, 39). The fact that gonadotrophic hormone stores in the pituitary can be decreased even in lesioned rats with testes of normal size (7, 39) underscores the difficulty in interpretation of the relative importance of the alleged separate gonadotrophic centers in regulation of gonadotrophic hormone secretion. Classic neuro-anatomic technics may have to be employed to evaluate more precisely the extent of damage in the hypothalamus, even in apparently unaffected areas.

The failure to induce impressive adrenal atrophy in the female rat after electrocautery of the hypothalamus has been general (2, 16, 17, 18, 34). In the male, marked adrenal atrophy can be produced with median eminence lesions (27) but it is usually not selective, being associated frequently with pan-hypopituitarism (6, 7, 39). The absence of morphologic change in the adrenal does not preclude the strong probability that some impairment of adreno-cortical function occurred in untreated lesioned rats of this study. McCann and his associates have demonstrated that median eminence lesions can block acute responses of the adrenal cortex without atrophy (25), will suppress compensatory hypertrophy of the gland to unilateral adrenalectomy (26) and may inhibit the rise in ACTH content of blood and pituitary which attends adrenalectomy (27, 28). The fact, however, that adreno-cortical hypertrophy can be induced with such stressful procedures as starvation or chronic thyroid hormone treatment—both potent activators of the pituitary-adrenal system (40, 41)—strongly suggests that the pituitary can augment its basal ACTH secretion even after extensive hypothalamic lesions. It has also been shown, as in previous work (5, 25), that lesion-induced diabetes insipidus and adrenal hypertrophy are not mutually exclusive. Evidence of adreno-cortical stimulation in rats with severe neuro-hypophysial damage indicates at best a limited role of the neurohypophysial hormones in facilitating SUSTAINED ACTH release by the anterior pituitary, a conclusion equally applicable to TSH function as well (11, 12).

The finding that exogenous thyroid hormone engenders adrenal hypertrophy without reversing the gonadal atrophy reveals that the effects of hypothalamic damage on the target endocrine glands are not merely nonspecific consequences of lowered metabolism. Brooks, Marine and Lambert (42) induced hypermetabolic states in lesioned rats with thyroxine but did not study the histo-physiologic consequences to the endocrine system. As in their study, thyroxine administration was found to retard but not abolish the weight gain in operated animals. The stimulation of the reproductive tract in the intact rat receiving thyroxine or triiodothyronine cannot be satisfactorily explained. Maqsood (29) and Reineke and Soliman (30) have evaluated the many discrepant reports which nevertheless strongly implicate the thyroid gland in mammalian reproductive processes. Experimental procedures, which in themselves drastically altered thyroid hormone levels far beyond physiologic limits, have tended to obscure the possible role of the thyroid in gonadal function. In the present study, this factor was taken into account. With hyperthyroid states ranging from mild to moderate and severe, there was marked stimulation of the reproductive tract in the intact rat. The effects on the uterus and vagina were much like those induced with chronic estrogen administration.

Several physiologic mechanisms exist through which thyro-ovarian interaction may conceivably occur; namely, 1) an indirect effect mediated via the adrenal, 2) a change in excretion rates of gonadotrophic and ovarian hormones or altered sensitivity of target tissues to them, and 3) a direct effect of thyroid hormone on the pituitary-ovarian axis with or without involvement of the hypothalamus. Inasmuch as the adrenal hypertrophy in operated rats was equivalent to that of intact controls, it is not deemed likely that differential response of the ovary was due to differences in adrenocortical hormone levels. Differential excretion or inactivation of gonadotrophic hormones and threshold differences in the gonad are known to occur in hyperthyroid rats (43). That such changes were restricted to the intact and not the lesioned animal despite similar thyroid hormone treatment over a range of dose levels is implausible. More relevant may be the fact that thyroid hormone in amounts inducing relatively mild hyperthyroidism is known to augment certain phases of gonadotrophic hormone secretion by the pituitary (29, 44). The substantial decrease in the numbers and cytologic activity of the delta basophils in the adenohypophyses of untreated lesioned rats and their considerable increase in the pituitary of the unoperated animal receiving thyroid hormone are consistent with this thesis. The persistence of gonadal atrophy in the operated animal could reflect irreparable damage to the mechanisms of adenohypophysial FSH and LH secretion. The restoration of delta cells in the adenohypophysis with exogenous thyroid hormone renders this interpretation questionable, however, and suggests further that thyroid hormone can act at both hypothalamic and pituitary levels. The reappearance of these cells at a time when TSH content of the anterior pituitary is far below normal excludes them as the producer of TSH (12) but whether their return signifies production of gonadotrophins without concomitant release, or even augmented ACTH secretion in view of the adrenal enlargement, cannot be resolved with the data at hand. The influence of the thyroid on the hypothalamic- hypophysial-ovarian system must therefore remain speculative at present.

Acknowledgment

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THE EFFECTS OF EPINEPHRINE ON THYROXINE METABOLISM: IODINE EXCRETION AFTER VARIOUS THYRONINE DERIVATIVES[1]

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ABSTRACT

Epinephrine was found to increase urinary I^{131} (primarily as iodide) after the administration of labeled L-thyroxine, D-thyroxine and L-triiodothyronine as compared to the I^{131} excretion after injection of these thyronines without epinephrine. Epinephrine had no effect on the excretion of isotope after the administration of radioactive iodide. Fecal I^{131} from these sources was slightly lowered by epinephrine. Other thyronine derivatives were studied without epinephrine and were found to have varying rates of excretion.

SWANSON has shown that the increased oxygen consumption produced in rats by epinephrine is thyroid-hormone-dependent (1). This effect is of short duration, oxygen consumption returning to normal within four hours. It appeared possible that this effect is related to an epinephrine effect on the metabolism and/or distribution of thyroid hormone. It has been shown that epinephrine produces a lowering of the serum protein bound iodine in rats and humans (2, 3, 4, 5).

In the present study the effects of epinephrine on iodine excretion were determined. Thyronine derivatives labeled with I^{131} were administered to thyroidectomized rats which had been withdrawn from replacement therapy (3.5 μg. L-thyroxine/100 gm. body weight/day) one month prior to the start of these experiments. This was done to lower the body pool of thyroid hormone to a level at which the injected labeled material would represent essentially all the thyroid hormone available to the organism.

Since metabolic rates were not determined, it is not known if the amounts of thyroactive compounds used in this work (2–4 μg.) supported the stimulatory effect of epinephrine on oxygen consumption. Although Swanson found increased oxygen consumption after epinephrine administration to rats receiving daily injections of 3 μg. of thyroxine, it was not the purpose of the experiments reported here to necessarily cause hypermetabolism. It was assumed that, even in the absence of a demonstrable effect on oxygen consumption, an effect of epinephrine on thyroid hormone metabolism would be exerted on thyroactive material administered as a single dose.

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In addition to the studies utilizing epinephrine, the excretory patterns of radioactive tetraiodothyroacetic (Tetrac) and tetraiodothyropropionic (Tetraprop) acids and of triiodothyroacetic acid (Triac) were determined.

MATERIALS AND METHODS

Male Long-Evans rats which had been thyroidectomized with I^{131} were used.[2] Groups of five rats were given a single intraperitoneal injection of the thyronine derivatives in amounts of 2–4 μg., incorporating 75–85 μc of radioactivity.[3] The animals were then placed in metabolism cages and urine and feces were collected over 24 or 48 hour intervals for varying periods of time.

The urine was pooled, filtered and made up to a standard volume. The feces were also pooled, homogenized and made up to volumes. Radioactivity in aliquots of both urine and feces was determined in a scintillation well counter. Total counts in the pooled urine and feces were compared to those in a standard containing the amount of radioactive material given to each rat.

In the studies utilizing epinephrine,[4] it was administered intramuscularly at a dose of 80 μg. per 100 gm. body weight. This single injection was made immediately after the administration of labeled thyroid hormone. Compounds which were studied both with and without epinephrine were given to the same group of five animals in both experiments.

Four rats were given I^{131} -iodide (60 μc). After 48 hours two of these animals were given epinephrine. Urine and feces of the four rats were collected during the initial 48 hours; they were collected as two pools for the subsequent 24 hours.

In some of the studies additional aliquots of urine and feces were acidified and extracted three times with equal volumes of n-butanol. The pooled butanol was then washed twice with Blau's reagent (4N NaOH containing 5% Na_2CO_3), which extracts iodide and iodinated tyrosines but not iodinated thyronines. Radioactivity in the butanol was determined before and after Blau's extraction.

RESULTS

The amounts of urinary and fecal radioactivity are tabulated in Table 1. Increased urinary I^{131} was found after epinephrine administration with L-thyroxine, D-thyroxine and L-triiodothyronine but not with iodide. Epinephrine produced a slight decrease in fecal I^{131} with the three thyronine derivatives.

Urinary I^{131} was primarily in the form of iodide; fecal I^{131} behaved mainly as thyronine derivative in its solubility characteristics. Fecal excretion was very low (<4%) in all the rats given isotopic iodide. Table 2 contains the results of butanol and Blau's reagent extractions; Blau's insoluble material has been recorded as "thyronine-like."

The urinary excretion of isotope was greater with triiodothyronine and D-thyroxine than with L-thyroxine. This was true also of Tetraprop and

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[2] Two treatments of 500 μc were used; histological examination of similarly treated rats showed no thyroid tissue in the throats or chests.

[3] Radioactive materials were purchased from Abbott Laboratories, Oak Ridge, Tennessee.

[4] "Adrenaline-in-oil," 1:500, Parke, Davis and Co.

TABLE 1. URINARY AND FECAL I^{131} [1]

Material injected	Urinary I^{131}								Fecal I^{131}							
	Time (hours)								Time (hours)							
	24	48	72	96	120	132	168	Total	24	48	72	96	120	132	168	Total
L-thyroxine	14	8		5				27		43		12				55
L-thyroxine & epinephrine	20	11	10	8				49	21	13	9	4				47
D-thyroxine	29	10	3					42	34	12	2					48
D-thyroxine & epinephrine	34	19	5					58	28	7	3					38
Triiodothyronine	25		15					40	46		15					61
Triiodothyronine & epinephrine	47	25						72	29	9						38
Triac	25	5	2	1				33	41	10	1	1				53
Tetrac	9	4		4	2		3	22	6	7		10	5		8	36
Tetraprop	18	8	4			11		41	11	17	12			18		58
Iodide	61	11	5					77	2	1	<1					<4
Iodide & epinephrine[2]			4					76			<1					<4

[1] Data recorded as per cent of administered radioactivity Each figure represents the average excretion per rat as calculated from the pooled excreta of five rats.

[2] Epinephrine administered at 48 hours

triac. Only tetrac was deiodinated more slowly than L-thyroxine. The increase in urinary radioactivity over the entire collection period following epinephrine was, with D-thyroxine—38%, with L-triiodothyronine—80% and with L-thyroxine—82%. Radioactivity derived from triiodothyronine was completely excreted within 48 hours when epinephrine was given. A longer collection period was required for essentially complete excretion with epinephrine and the two thyroxine isomers.

TABLE 2 PERCENTAGE OF THYRONINE-LIKE I^{131} IN BUTANOL EXTRACTS

Material injected	Urine	Feces
L-thyroxine	2	90
L-thyroxine & epinephrine	3	83
D-thyroxine	1	83
D-thyroxine & epinephrine	2	79
Tetraprop	2	86
Triac	1	58
Iodide	2	—

DISCUSSION

From the data presented above, it appears that the rates of deiodination of the various thyronine derivatives are not related to their *in vivo* activities. Thus, Tetraprop, triac and D-thyroxine, which are relatively inactive in mammals, were deiodinated more rapidly than L-thyroxine. The more active compound, L-triiodothyronine, was also deiodinated more rapidly than L-thyroxine. Tetrac, a less active material, was deiodinated more slowly than L-thyroxine.

Epinephrine effected a marked increase in urinary I^{131} with the three thyronine derivatives tested. It did not increase the excretion of isotope when given 48 hours after the administration of radioactive iodide, indicating that the epinephrine effect is not exerted on the release of iodide from the tissues. Furthermore, the increased urinary I^{131} after thyronines and epinephrine persisted for 48–72 hours. It is questionable if an epinephrine effect on renal action would persist for this length of time.

The decrease in fecal iodine after epinephrine may result from the lowered concentration of circulating labeled material. Specific differences in the rate of biliary excretion may exist, however. Thus, Tetrac was excreted at a low rate in both urine and feces. Triac, on the other hand, was rapidly excreted via both routes.

Further work on this problem will be designed to determine the mechanism by which epinephrine increases the urinary excretion of iodide derived from thyronine derivatives.

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CHARACTERIZATION OF ESTROGENS IN THE BOVINE[1,2]

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ABSTRACT

Estrone, estradiol-17β and estradiol-17α were identified in bovine placental extracts by chromatography on paper, formation of acetates, Kober reaction, bioassay and absorption spectra in sulfuric acid. A $FeCl_3$-$Fe(CN)_6$ reacting compound with a migration rate on paper chromatograms similar to estradiol-17β was detected in extracts of ovaries from non-pregnant cows but in quantities too low for further identification.

Maternal adrenals and ovaries and fetal adrenals and testes from two cows pregnant 256 and 265 days did not yield detectable quantities of estrogens. The placenta appears to be the principal source of estrogens in the pregnant bovine.

INTRODUCTION

INFORMATION on the natural estrogens of the bovine is limited to identification of estrone in adrenal tissue (1), estrone and estradiol-17α in urine from pregnant cows (2, 3) and estrone in the bile of pregnant cows (4). Nonspecific assays of urine by biological (5) and fluorometric (6) methods have indicated that estrogen levels are markedly increased in the pregnant cow as compared with the non-pregnant cow.

Estradiol-17β and estrone have been demonstrated in the sow ovary (7, 8). The principal source of estrogens in pregnant women is considered to be the placenta from which estrone, estradiol-17β, and estriol have been isolated (9, 10, 11).

The purpose of this study was to determine the identity and principal sources of the estrogens in the bovine.

MATERIALS AND METHODS

Tissue Sources

Ovaries from nonpregnant cows and heifers with grossly normal reproductive tracts were obtained within 15 minutes after slaughter. The ovaries were pooled in groups of 8, 11, and 35 ovaries and assayed by the procedures described below.

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Two Jerseys were sacrificed during late pregnancy. No. 2223 was pregnant 256 days with her first calf and No. 2192 was pregnant 265 days with her third calf. Placentae, ovaries, and adrenals of the cows and the adrenals and testes of their fetal calves were removed for assay.

Placentae were obtained at slaughter from cows in different stages of gestation and from cows following parturition.

Chemical Procedures

All tissues were homogenized and extracted with ethyl acetate in a Waring blendor. The lipid extract was evaporated to dryness in a water bath at 45° C under reduced pressure. Fats were removed by taking up the lipid residue in 70% methanol and then cooling overnight at minus 5 to 10° C. The precipitated fat was removed by centrifugation or filtration in the cold. In the case of samples from cow No. 2192 and a few placental extracts, fat was removed by partitioning between 70% methanol and Skellysolve B. The aqueous fraction, after evaporation of the methanol in a water bath at 45° C under a stream of N_2, was extracted with ethyl acetate. The ethyl acetate was dried over Na_2SO_4 and evaporated to dryness in a water bath at 45° C under a stream of N_2. The resulting residue was chromatographed on paper in the formamide/Skellysolve B system (12) for 4 to 5 hours. The chromatograms were dried in air and the areas within 4 cm. of the origin eluted with methanol. After evaporation of the methanol, the residue was chromatographed in formamide/Skellysolve B-benzene for 5 to 6 hours. After drying the chromatogram, the estrogens were detected with $FeCl_3$-$K_3Fe(CN)_6$ reagent. The origin was eluted as described above and rechromatographed in the benzene/55% methanol system for 24 hours in order to detect estriol if present.

Identification Procedures

When sufficient quantities of a compound were available, the compounds were rechromatographed for various periods of time in formamide/Skellysolve B-benzene. Monoacetates were formed by reacting with one part acetic anhydride to 50 parts pyridine (13). The acetylated estrogens were chromatographed in formamide/Skellysolve B till the solvent front had moved approximately 40 cm. The acetylated estrogens were detected on the chromatograms by the procedure suggested by West (14). Kober tests were run with Hunt's (15) microadaption of the Brown (16) procedure. Four point bioassays, using the 6-hour uterine weight method of Astwood (17) were calculated with covariance as suggested by Bliss (18). Absorption spectra were determined after reacting the compounds with concentrated sulfuric acid for 2 hours. Although this study was not a quantitative one, crude estimates were made by visual comparisons of the dyed spots on chromatograms from tissue extracts and standards.

RESULTS

Up to 35 ovaries in a group were extracted but no estrogen could be completely identified. In all cases, a faint spot was detected on the chromatograms corresponding to estradiol-17β. The quantities were less than 10 μg. and insufficient for further identification. No other phenolic compounds were detected in the ovarian extracts.

Three $FeCl_3$-$Fe(CN)_6$ reacting compounds were detected in the placentae from the two Jersey cows sacrificed during late pregnancy. No phenolic compounds were detected in the ovaries and adrenals of these cows, nor was any found in the adrenals and testes of their fetal calves.

$FeCl_3$-$Fe(CN)^6$ reacting compounds from placental extracts were pooled for further identification. Estrone was identified by migration rate on paper chromatograms, by migration rate of the acetate, by Kober chromogen, and by a significant response in the uterine weight bioassay corresponding to authentic estrone.

Estradiol-17β was identified by migration rate on paper chromatograms, by migration rate of the monacetate, by Kober chromogen, and by significant response in the uterine weight bioassay parallel to authentic estradiol-17β at 1/10 the concentration of estrone.

Estradiol-17α was identified by migration rate on paper chromatograms (Fig. 1), by migration rate of the monoacetate, by Kober chromogen, and by sulfuric acid spectra of the monoacetate.

Estriol could not be detected in any of the extracts.

Table 1 shows the approximate quantities of the three estrogens from the placentae of the two pregnant cows. Relative amounts of estradiol-17α have been higher in term placentae. Estradiol-17β was detected in placentae from the other cows as early as the fifth month of pregnancy.

TABLE 1. APPROXIMATE[1] QUANTITIES OF THREE ESTROGENS IN TWO PLACENTAE

Cow	Tissue weight	Estrone	Estradiol-17β	Estradiol-17α
	gm.	μg.	μg.	μg.
2223	1400	20	30	5
2192	1300	15	30	10

[1] Visual comparisons from spots on paper chromatograms.

DISCUSSION

The placenta of the bovine, like that of the human, appears to be the principal source of estrogens during pregnancy. Estrone and estradiol-17β are found in the placentae of both species. The estrogens in the placentae differ, in that estradiol-17α but not estriol was found in the bovine. The reverse is true for the human (11). It is interesting that estradiol-17α is apparently a major urinary metabolite in the bovine (3) and estriol is considered the principal urinary metabolite in the human (19). Levy, cited by

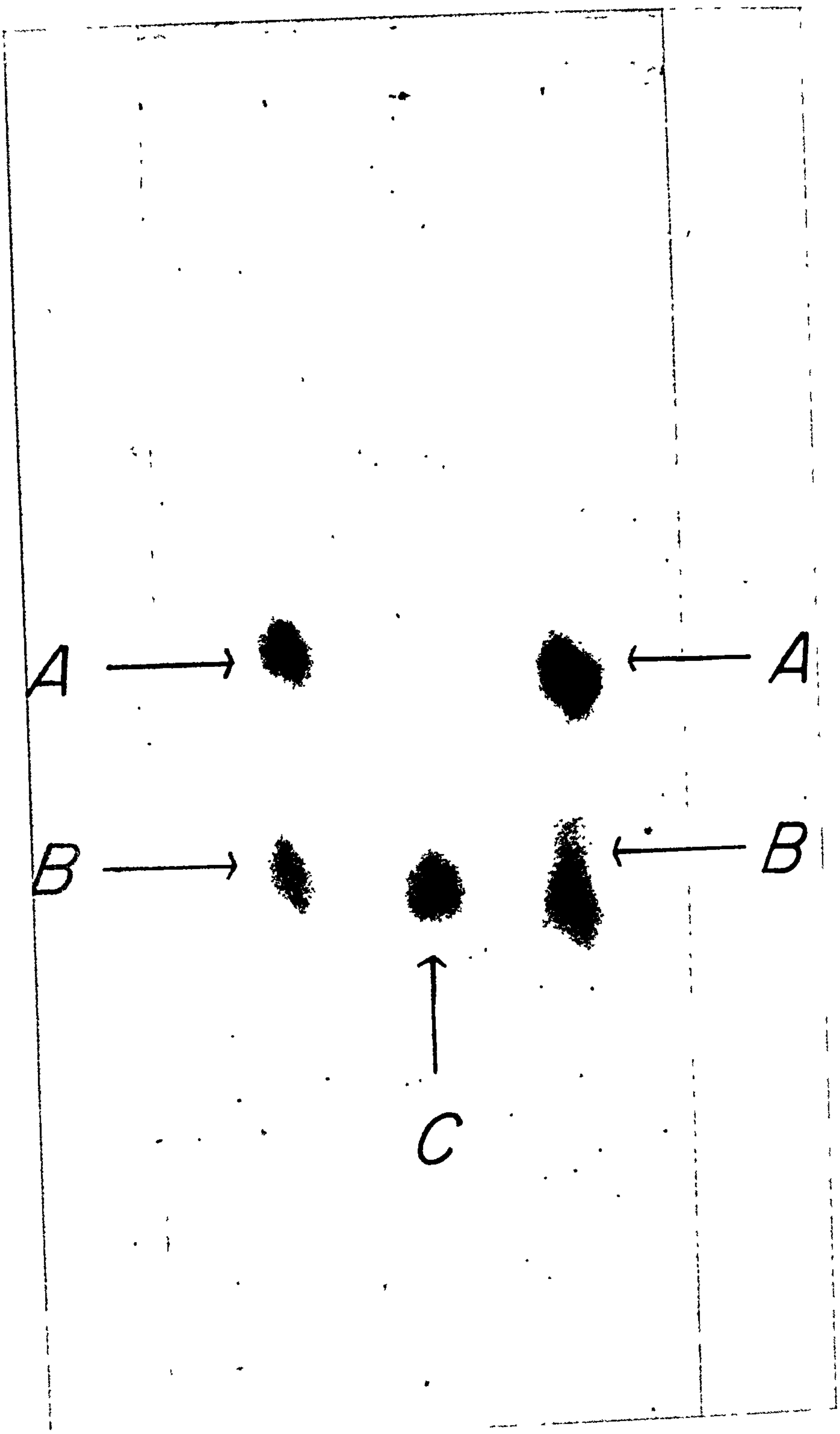

Fig. 1. Paper chromatogram after development with $FeCl_3$-$K_3Fe(CN)$ reagent. (A) Estradiol-17β standard, (B) Estradiol-17α standard, (C) Pooled placental estradiol-17α.

Dorfman (20), has reported that estrone is metabolized to estradiol-17α by bovine blood. This mechanism could account for a part or all of this compound in the highly vascular placental tissue.

A previous paper from this laboratory (21) confirms Short's (22) report that the bovine placentae does not contain progesterone or Δ^4 pregnene-20β-ol, 3-one. Also, testosterone and androstendione were not detected (21). One or more of these steroids, which are possible estrogen precursors, have been reported for the ovary, adrenal, testes, and human placenta. In light of this, bovine placental tissue might be of interest for studies of estrogen biosynthesis.

The low amount of estrogens in the bovine ovary is consistent with reports of low urinary excretion of estrogens in non-pregnant cows (5) and the relatively low amount of exogenous estrogen necessary to bring ovariectomized heifers into estrus (23). Although the data presented here are not conclusive, estradiol-17β is possibly the ovarian estrogen in the bovine.

Failure to detect estrogens in the maternal adrenal, fetal adrenal or fetal testis may be due to lack of sensitive enough methods but does indicate that these organs are minor sources as compared to the placenta.

The relatively high quantities of estrogens in late pregnancy placenta, coupled with rather constant levels of progestins in the ovaries (21) and blood (24) of the bovine, is in contrast to the increasing levels of both groups of steroids in the human placenta during gestation (25, 26).

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FURTHER EVIDENCE FOR MEDIATION BY HISTAMINE OF ESTROGENIC STIMULATION OF THE RAT UTERUS[1,2]

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ABSTRACT

Solutions of histamine dihydrochloride or of a synthetic histamine releasing agent (Compound 48–80) were introduced into the uterine lumen of adult, castrated rats. Within four hours following administration, increases in water content and changes in gross vascular appearance of the organ were produced which were comparable to the maxima induced by intravenous or intraluminal estrogen for the same period of treatment. Intravenously administered cortisol substantially diminished the hyperemia and water imbibition elicited by 48–80 or by histamine.

Two of the 4 structurally interrelated antihistamines tested (Benadryl hydrochloride and Chlor-Trimeton maleate), were effective in blocking estradiol-stimulated hydration and hyperemia.

These findings are regarded as support for the view that estrogen action, in effecting vasodilation and edema in the rat uterus, is mediated by the local release of histamine. It is proposed that cortisol may antagonize responses to estrogen in the uterus by non-specifically decreasing capillary permeability in that organ.

IN EXPERIMENTS reported earlier from this laboratory (1), it was observed that a single intravenous dose of estradiol-17β administered to the ovariectomized rat was followed in 4 hours by a decrease in the histamine concentration of the uterus. The loss was determined to be approximately 17% of the basal concentration in the organ and was shown to be neither a consequence of the usual water accumulation nor a reflection of an estrogen-induced acceleration of histaminase activity. In addition, uteri taken from intact animals during periods of high endogenous estrogen secretion exhibited less histamine than those in quiescent states. On the basis of these observations it was proposed that certain early correlates of estrogen stimulation, particularly vasodilation accompanied by increased

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capillary permeability, may be attributed to the action of histamine released *in situ* by estrogen.

Results of experiments designed to test further this hypothesis are included in the present report. It has been demonstrated that instillation of histamine or of a histamine liberator, Compound 48–80, into the uterine lumen of the ovariectomized rat simulates the action of estrogen in eliciting hyperemia and hydration of the organ. When induced by estrogen itself, these responses could be significantly depressed by administration of the antihistamines, Benadryl hydrochloride and Chlor-Trimeton maleate. Cortisol administration suppressed the vascular and hydration responses elicited in the uterus by histamine and 48–80.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats, 4 to 7 months of age and averaging 300 gm. in weight, were used. All animals were bilaterally ovariectomized 14 to 16 days before treatment. In one experiment the animals were also bilaterally adrenalectomized just prior to injection. Water and Purina laboratory chow were fed *ad libitum*. While the animals were under light ether anesthesia, solutions of diphenhydramine ("Benadryl")-HCl, pyranisamine ("Neo-Antergan") maleate, tripelennamine ("Pyribenzamine")-HCl or chlorprophenpyridamine ("Chlor-Trimeton") maleate, Compound "48–80," histamine dihydrochloride, estradiol-17β, or their control solutions were injected directly into the lumen of one or both uterine horns. Ligatures were placed immediately above the site of injection. Although varying concentrations of the above substances were employed, the volume injected per horn was kept constant at 0.02 ml. This quantity of fluid did not discernibly distend the uteri of most of the animals used.

In the antihistamine experiments, estradiol-17β in isotonic saline solution (2) was administered intravenously in the amount of 0.5 μg. (0.5 ml.) per 100 gm. body weight unless otherwise stated. From 2.5 to 4.0 mg. of cortisol acetate in aqueous suspension (25 mg./ml.) was administered per 100 gm. body weight to some of the animals receiving intraluminal histamine or Compound 48–80. The hormones were given under light ether anesthesia, each in a single dose via the saphenous vein immediately preceding intrauterine instillation of the non-steroid substances mentioned.

At time intervals following injection, each animal was stunned by a sharp blow at the base of the skull and bled by decapitation; uterine horns were removed (1) and analyzed for water content as previously described (2).

RESULTS

Compound 48–80 and uterine hydration

Naturally occurring and synthetic materials possessing the capacity to cause the release of endogenous histamine have been extensively studied by the pharmacologists (3). Among the more powerful of these is a substance designated Compound 48–80 (3, 4), the exact structure of which is unknown but is believed to consist of molecular chains of an indeterminate number of P-methoxy-N-methyl-phenylethylamine units. Injection of this material at various dose levels directly into the lumen of each uterine horn resulted 4 hours later in the increases in uterine water uptake shown in Figure 1. A dose of 0.2 mg./horn can be seen to produce a water uptake

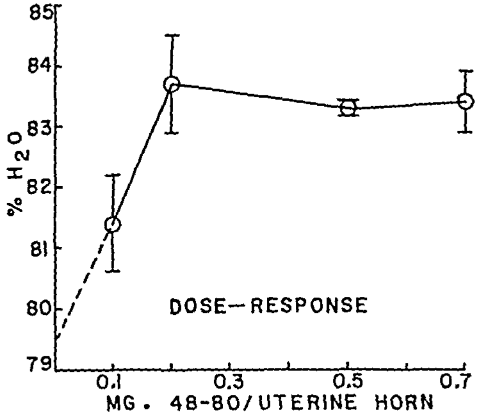


FIG. 1. The effect on uterine hydration of increasing amounts of 48-80 injected directly into the lumen of each uterine horn. Each point represents the mean ± the standard deviation of values obtained from 4–5 animals sacrificed 4 hours after injection.

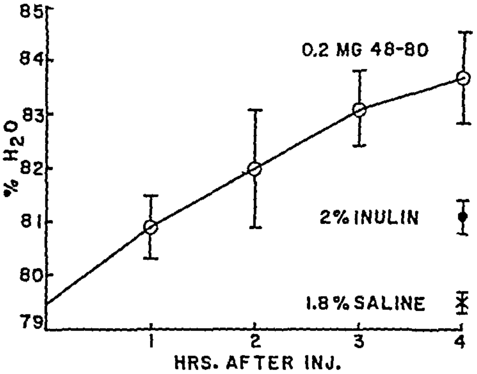


FIG. 2. The influence of time after administration of Compound 48-80 on uterine water content. The histamine releaser was injected intraluminally in the amount of 0.2 mg. per uterine horn. Values for inulin and saline given by the same route are shown for the 4 hour time period. Each point represents the mean ± the standard deviation of values obtained from 4–6 animals.

approaching the maximum attainable with intravenous estrogen (*vide infra*). The local route of administration was employed because 48–80 was observed in preliminary experiments to be toxic when given by systemic routes. Intra-uterine dosages in excess of 0.2 mg./horn were increasingly toxic to the animals generally and also caused extensive vascular congestion in the uterus. Uterine horns exposed to 0.1–0.2 mg., however, displayed a gross appearance indistinguishable from those of intact animals in proestrus, or of ovariectomized rats given estrogen.

A time study utilizing the 0.2 mg. dose of 48–80 is depicted in Figure 2. Inulin was utilized in this experiment as a control for osmotic effects that might result from intraluminal administration of a complex substance such as 48–80. It may be noted that inulin at the 4 hour level caused a water uptake which was, however, significantly below that produced by 48–80 at the same time interval. Thus, a portion of the hydration produced by the histamine releaser may be an osmotic effect of the concentrations employed. Nevertheless, uteri injected with 2% inulin or 1.8% saline showed no hyperemia or gross edema, in contrast to those exposed to 48–80.

An effort was made to block with cortisol the uterine hydration induced by Compound 48–80. Concomitant administration of intra-uterine 48–80 (0.2 mg./horn) and of 3.5 mg. of cortisol acetate per 100 gm. of body weight via the saphenous vein resulted 4 hours later in a significant suppression ($P < 0.01$) of the water uptake observed in the presence of the histamine liberator alone (Fig. 3). For purposes of comparison, data depicting the hydration response to intravenous estradiol (0.5 μg./100 gm. body weight) have been included in Figure 3. It should be mentioned that the same degree of edema at 4 hours was obtained by introducing only 0.02 μg. (in 0.02 ml.) estradiol-17β into the lumen of the uterus (not shown in Figure).

Exogenous histamine and uterine hydration

Since local liberation of endogenous histamine through the action of Compound 48–80 elicited uterine hyperemia and water imbibition, it was deemed advisable to study the capacity of exogenous histamine to evoke a similar response. Accordingly, varying amounts of histamine dihydrochloride were injected into the lumen of each uterine horn and the tissue assayed for water content 4 hours later. One mg. histamine/horn produced a response maximum comparable to that induced by intraluminal 48–80 and approaching that elicited by 0.5 μg. of estradiol/100 gm. body weight, I.V. (Fig. 4). Cortisol acetate (2.5 mg./100 gm. body weight, I.V.) given at the same time as the histamine depressed the histamine-induced water uptake to near control levels (Fig. 5).

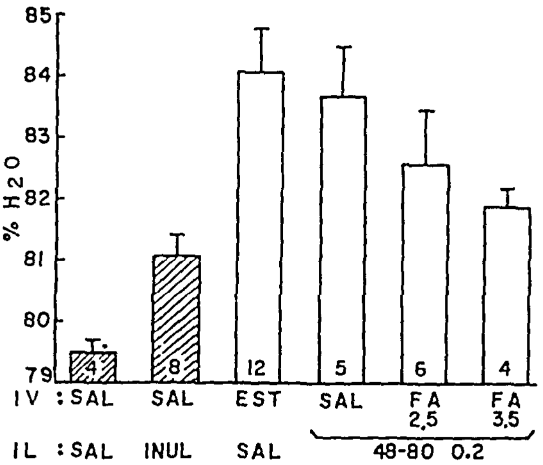


FIG. 3. The effect of cortisol on the uterine hydration due to Compound 48-80. Saline and inulin controls are shown crosshatched. Materials were injected both intravenously (IV) and also directly into the lumen of each uterine horn (IL). Numbers beneath the bars are dosages in mg./100 gm. body weight for cortisol acetate (FA) and mg./horn for 48-80. Saline concentrations employed were 0.9% (IV) or 1.8% (IL); inulin concentration, 2%; estradiol dosage (EST), 0.5 μg./100 gm. body weight. The height of each bar represents the average value resulting from the number of determinations indicated by the enclosed figure. Positive portions of the standard deviations are shown.

The influence of antihistamines on estrogen-induced uterine water uptake

A number of pilot studies had indicated that none of the 4 antihistamines tested was effective in altering the uterine response to estrogen when the former were given intravenously or intraperitoneally and under a variety of conditions of pretreatment and dosage. When administered locally into the uterus, however, 2.0–5.0 mg./horn of Chlor-Trimeton (Table 1) or 2.5–5.0 mg./horn of Benadryl were found to be effective in substantially inhibiting water imbibition due to estrogen. The inhibitory action of Chlor-Trimeton was not mediated by increased secretion of anti-inflammatory

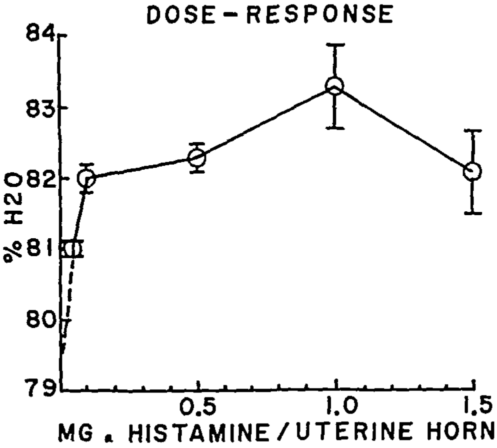


FIG. 4. The effect on uterine water content of increasing amounts of histamine dihydrochloride injected into the lumen of each uterine horn. Each point represents the mean ± the standard deviation of values obtained from 4 animals sacrificed 4 hours after injection.

adrenocorticoids since the antihistamine was equally effective in the absence of the adrenals (Table 1).

Pyribenzamine and Neo-Antergan were ineffective in the above mentioned dosages. The toxicity of these last named compounds was such as to preclude testing in amounts greater than 5.0 mg./horn. The antihistamine experiments involving intrauterine administration over the 2.5–5.0 mg. range are summarized in Figure 6.

Benadryl (I) and Chlor-Trimeton (II), found active in blocking the water response, differ structurally from Pyribenzamine (III) and Neo-Antergan (IV), found ineffective, in that the former group does not contain the intercyclic N atom common to the latter (Fig. 7).

pH of substances administered

At concentrations employed in the experiments, histamine and 48–80 were in the acid range (Table 2). The hyperemia and edema which resulted

TABLE 1. THE INFLUENCE OF CHLOR-TRIMETON MALEATE ON UTERINE WATER IMBIBITION IN ADULT, OVARIECTOMIZED RATS 4 HOURS FOLLOWING ESTROGEN ADMINISTRATION

Experimental conditions	No. of determinations	% uterine water
A. *Saline*—0.5 ml. isotonic/100 gm. body wt., I.V.		
Saline, 0.02 ml. (1.8% instilled into lumen of each horn at same time as I.V. saline.	4	79.5 ±0.2†
B. *Estradiol-17β*—0.5 μg./100 gm. body wt., I.V.		
Solution instilled into lumen of each horn at same time as intravenously administered estrogen:		
Saline, 0.02 ml. (1.8%)/horn	12	84.1 ±0.7
Chlor-Trimeton, 0.5 mg. (0.02 ml.)/horn	4	83.7 ±0.4
Chlor-Trimeton, 1.0 mg. (0.02 ml.)/horn	4	84.2 ±0.6
Chlor-Trimeton, 2.0 mg. (0.02 ml.)/horn	5	82.6 ±0.8*
Chlor-Trimeton, 2.5 mg. (0.02 ml.)/horn	4	82.0 ±0.9**
Chlor-Trimeton, 5.0 mg. (0.02 ml.)/horn	4	81.4 ±1.7*
Adrenalectomized just before injections:		
Chlor-Trimeton, 2.5 mg. (0.02 ml.)/horn	4	81.4 ±0.5**

* $P < 0.01$, ** $P < 0.001$ compared to the I.V. estradiol-intraluminal saline group.

† Mean ±standard deviation.

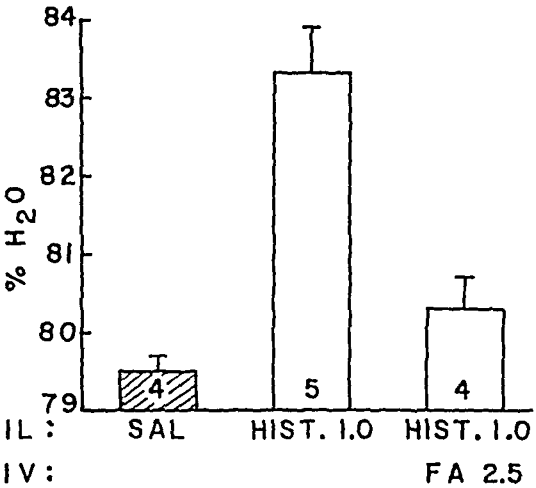


Fig. 5. The influence of intravenous cortisol on the increase in uterine water produced by the intrauterine administration of histamine. Numbers beneath the bars refer to dosage in mg./100 gm. body weight for cortisol acetate (FA) and mg. of the dihydrochloride per uterine horn for histamine (HIST.). Intraluminal saline (SAL) as in Figure 3. The height of each bar is determined by the average value resulting from the number of determinations indicated within the bar. Positive portions of the standard deviations are shown.

from the administration of those compounds cannot, however, be attributed to an acid environment since injection of phthalate-HCl buffer pH 3.1 (Table 2) produced no significant hydration changes from routinely observed control levels in the organ (cf. Figs. 1–6; Table 1). Furthermore, the two most acidic antihistamines were those found to be active in *inhibiting* the water inbibition due to estrogen.

DISCUSSION

Earlier data presented from these laboratories revealed that uterine histamine concentration in the rat is under estrogenic control (1). Depletion of histamine *in situ* was found to be associated with the onset of hyperemia and the imbibition of water in the uterus of the ovariectomized rat treated 4 hours earlier with estradiol-17β. It was further shown that endogenous estrogen secretion in the intact rat was similarly capable of exerting a histamine-liberating influence in the uterus (1).

Table 2. pH of intraluminally administered agents

Compound	Concentration, mg. per 0.02 ml.[1]	pH	Effective in depressing uterine H_2O response to estradiol	% uterine H_2O
Pyribenzamine HCl	2.5	6.4	−	
Neo-Antergan maleate	2.5	5.2	−	
Chlor-Trimeton maleate	2.5	5.0	+	
Benadryl HCl	2.5	3.9	+	
Histamine dihydrochloride	1.0	3.8		83.3 ± 0.6[2]
48-80	0.2	3.1		83.7 ± 0.8
Phthalate-HCl buffer	0.02 ml.	3.1		80.6 ± 0.6[3]

[1] Concentration and volume instilled into each uterine horn in experiments described in the text.

[2] Mean ± standard deviation.

[3] $P < 0.001$ (compared to the value for histamine or 48-80).

The present report extends these findings and provides additional support for the view proffered earlier (1) that the acute changes in the vascularity and capillary permeability of the uterus in response to estrogen are mediated by the histamine liberated *in situ* in the presence of the hormone. Histamine or a histamine liberator (Compound 48–80) administered by uterine instillation promotes striking estrogen-like effects in the absence of the hormone. Thus, local vasodilation, hyperemia and water imbibition, characteristic early uterine responses to estrogen action, have been reproduced under these conditions.

These data strongly suggest participation of histamine or related materials at a primary stage in the action of estrogen at the target organ. In this connection a possible role for other naturally occurring substances with similar functions should not be excluded from consideration. Serotonin (5-hydroxy-tryptamine), for example, promotes hyperemia and edema formation, especially in the rat, and is depleted from tissues along with histamine by "liberators" of the latter (3, 5).

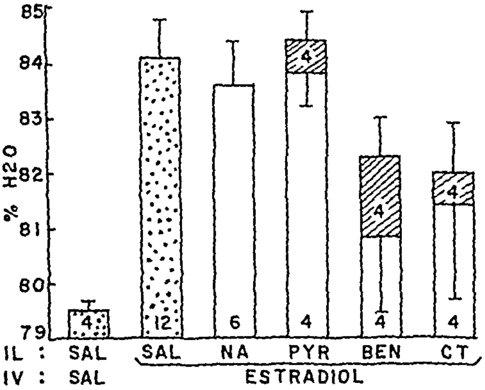
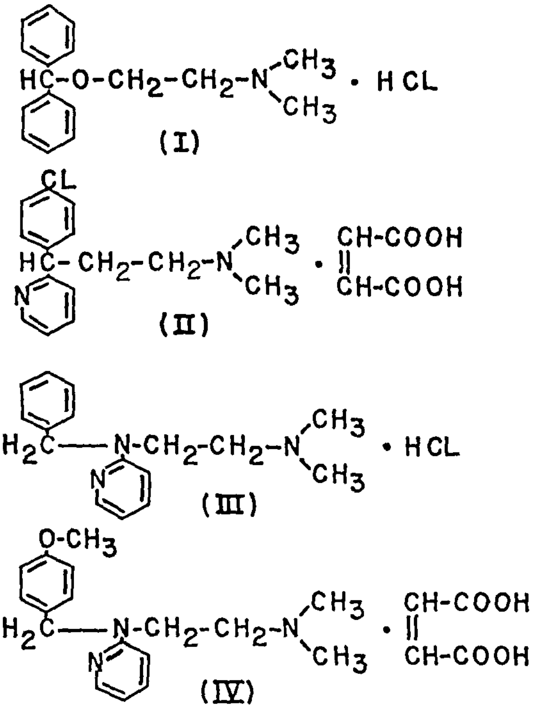


FIG. 6. The influence of antihistaminic compounds on the uterine water response to intravenous estradiol. Antihistamines were given 4 hours before sacrifice directly into the lumen of each uterine horn (IL). Stippled bars represent saline or estradiol controls in dosages and concentrations indicated in Figure 3. In the remaining bars, the total height (clear plus cross-hatched portions) of each represents the effect on uterine water of 2.5 mg./horn of the antihistamine indicated; clear portion only, response to 5.0 mg./horn. Na refers to Neo-Antergan maleate; PYR, Pyribenzamine HCl; BEN, Benadryl HCl; CT, Chlor-Trimeton maleate. The number of determinations for each group appears within each bar. Positive or negative portions of the standard deviations are shown.

Isolated findings, suggestive of local histamine mediation in uterine responses to estrogen, have appeared in the literature from time to time. Holden (6) had suggested in 1939 that estrogen action may be mediated by a histamine-like substance when he found that histamine relieved uterine vasoconstriction produced by a variety of agents. Daily parenteral injections of 200 μg. of histamine for 8 days produced in the vagina of the intact rat a histological picture characteristic of pro-estrus and estrus (7). Similar results were reported by Champy, Coujard and Demay (8) on chronic instillation of epinephrine, histamine or choline directly into the vagina of guinea pigs. Intra-uterine administration of histamine in the rabbit induced morphological changes typical of those elicited by estrogen treatment in the presence of progesterone (9). Given with estrogen over a 5 day period, histamine or acetylcholine enhanced the effect of the steroid in producing an increase in uterine weight (10). Evidence militating against a role for acetylcholine as a natural mediating substance in responses to estrogen has been summarized elsewhere (1, 11).

The blockade of estrogen-stimulated hyperemia and water accumulation by antihistaminic compounds demonstrated in the present studies is consistent with the postulated function for histamine in mediating estrogen action. The significance of the apparent structural requirement for effective antagonism of these early responses to estrogen cannot be stated at this time.



HC-O-CH2-CH2-N CH3 CH3 • HCL
(I)
CL
HC-CH2-CH2-N CH3 CH3 • CH-COOH ‖ CH-COOH
N
(II)
H2C—N-CH2-CH2-N CH3 CH3 • HCL
N
(III)
O-CH3
H2C—N-CH2-CH2-N CH3 CH3 • CH-COOH ‖ CH-COOH
N
(IV)

FIG. 7. Structures of the antihistamines used in the studies summarized in Figure 6. Compound I is Benadryl HCl; II, Chlor-Trimeton maleate; III, Pyribenzamine HCl; IV, Neo-Antergan maleate. Compounds I and II were effective in blocking the uterine water response to estradiol whereas III and IV were ineffective in the dosages employed (see text).

Reports from other laboratories of experiments employing anti-histaminic compounds, support the present hypothesis. Neo-Antergan, given daily for 8 days into the intact rat, resulted in a quiescent vaginal mucosa characteristic of di-estrus-metestrus (7). Though ineffective when either was administered alone, Phenergan and an anti-cholinergic drug injected together prevented estrogen-stimulated estrus in ovariectomized rats (12). Also, Pyribenzamine diminished the potentiating effect of histamine on estrogen in the uterus (10), although that antihistamine was found to be ineffective in blocking the cyclic rhythm of blood flow in endometrial transplants (13).

Other studies have centered about the possible participation of histamine in the physiology of implantation and pregnancy. Shelesnyak has shown that antihistamines inhibit ovum implantation and the decidual response of pseudopregnancy (14), results also obtained in the rabbit by Chambon and Lefrein (15). Sulman (16) could not confirm the interference with nidation, however, in the mouse. Antihistamines were in addition found by Shelesnyak to interfere with normal pregnancy in the rat and mouse (14), whereas histamine applied topically to the rat uterus actually stimulated deciduomata formation without the necessity of mechanical traumatization (14, see also 9). Shelesnyak has postulated histamine participation in normal ovum implantation and formation of decidua (14). Any hypothesis implicating histamine in the initiation or maintainance of pregnancy, however, must eventually be integrated with the findings that histaminolytic activity was observed to be greatly increased in pseudopregnant uterus (17), in placenta (17, 18), and in plasma (19) and uterus (17) of pregnancy.

The 11-oxy, 17-hydroxycorticosteroids are known inhibitors of estrogen action *in vivo* (20, 21). Szego and Roberts have proposed two alternative mechanisms for this steroidal interaction (11, 21). Of these, competition by the two classes of steroid for sites on protein molecules essential for steroid hormone transport, or at the target organ itself, is supported by *in vitro* data (22, 23). The suggestion was also advanced by these workers that the cortisol-estrogen antagonism exhibited could well have been a reflection of the mutually opposing influences of these steroids on uterine permeability (11, 21, 24).

The present data, while they do not support or refute the competitive inhibition hypothesis, do provide support for the alternative mechanism of antagonism of the two steroids, at the level of some permeability phenomenon involving the uterine vasculature. Results presented elsewhere have shown that cortisol prevented the increases in sodium (25) as well as in fluid which normally occur (20, 21, 25) in response to estradiol alone. In the present studies, intravenously administered cortisol was also seen to diminish uterine hydration produced by local instillation of a histamine releasing agent or of histamine itself. In the light of these observations it would seem reasonable to suggest that cortisol does not interact directly with estrogen under the conditions described. Rather, it appears to do so secondarily by diminishing the vasodilation and accompanying edema attributable to the histamine released by estrogen. Such a mechanism would explain the finding that cortisol when administered concurrently with estrogen did not prevent the estrogen-induced release of histamine, although it did eliminate the other measured responses to estrogen (1). These views are consistent with findings that vasodilation (26, 27) and accompanying increase in capillary permeability (28, 29) and other reactions resulting from histamine liberation or injection in extra-uterine tissues, can be diminished or prevented by the administration of cortisone-like adrenal steroids (28, 30–35). Thus, cortisol appears to mimic the action of the effective antihistamines studied by antagonizing histamine at its site of action. The present results are therefore in harmony with what is known of the action of 11-oxy, 17-hydroxycorticosteroids in promoting a nonspecific increase in capillary resistance (33, 36–38).

It appears increasingly evident that histamine intrinsic to the uterus has one or more functions in the physiology of the pregnant and non-pregnant organ. The extent to which these functions may be interrelated is of fundamental importance. It is not unlikely that the vasodilatory property of histamine, leading to increased permeability of the capillary bed, is basic to all mechanisms involving the amine in the uterus.

Acknowledgments

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CORRELATION OF RADIOIODINE UPTAKE WITH THYROID WEIGHT IN THE MOUSE AT VARYING LEVELS OF EXOGENOUS AND ENDOGENOUS THYROTROPIN STIMULATION[1]

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ABSTRACT

Previous studies have shown that transplanting the pituitary to the anterior ocular chamber in mice alters its ability to stimulate the thyroid gland. Radioiodine metabolism is well maintained whereas thyroid weight falls to the hypophysectomy level. Chronic propylthiouracil treatment in such animals causes a marked increase in thyroidal radioiodine uptake but minimal increase in thyroid weight. In similarly treated normal controls both radioiodine metabolism and thyroid weight are greatly increased. These observations provided further evidence that separate "growth" and "metabolic" thyrotropins might exist, the growth factor requiring direct contiguity of the pituitary with the hypothalamus for adequate secretion.

Since it was possible that radioiodine uptake might be more sensitive to small amounts of a single thyrotropin than thyroid weight, the quantitative response of these two parameters to both exogenous and endogenous thyrotropin was compared. Radioiodine uptake was found to reach a maximum with a considerably smaller dose of TSH than did thyroid weight. The present results indicate that the data previously obtained in mice with heterotopic pituitaries can be explained within the classic framework of a single thyrotropin.

PREVIOUS studies in rats with hypothalamic lesions (1, 2, 3) and mice with heterotopically located pituitaries (4, 5) have indicated that these procedures alter the ability of the hypophysis to stimulate the thyroid gland. Radioiodine metabolism, as indicated by the thyroid/serum (T/S) iodide ratio and thyroidal radioiodine uptake, is well maintained whereas the ability of the thyroid to grow in response to chronic antithyroid drug administration is largely lost.

Investigation of the dichotomy thus produced suggested that there might be two separate thyrotropic factors secreted by the pituitary, one stimulating thyroidal iodine metabolism and one stimulating thyroid growth (2). The original studies in the rat showing this divergence of effects primarily utilized the T/S ratio as an index of iodine metabolism but subsequent work has shown that this parameter is affected to a considerable degree by factors other than TSH (6, 7). In addition, more recent studies

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in the rat have shown that certain hypothalamic lesions depress radioiodine uptake and thyroidal secretion rate as well as thyroid growth (8, 12). Thus evidence is accumulating that it may be unnecessary to postulate the secretion of more than one thyrotropin.

However, the changes produced in thyrotropic activity by transplanting the pituitary to the anterior ocular chamber in mice have not yet been satisfactorily explained on the basis of a single thyrotropin (4, 5). In such animals the thyroid and other target endocrine glands atrophy to the same extent as in completely hypophysectomized mice. In spite of the considerable reduction in thyroid size, the radioiodine uptake and T/S ratio of the animals with heterotopic pituitaries are maintained at an approximately normal level. Chronic propylthiouracil (PTU) administration, which presumably causes a greatly augmented secretion of TSH due to a lowering of circulating thyroxine, will result in augmentation of both the iodide-concentrating mechanism and organic binding of iodine (after stopping PTU for 2 days to allow its excretion). Mice with heterotopic pituitaries under these conditions have a considerably greater absolute level of radioiodine metabolism per unit thyroid weight than do similarly treated normal controls. In contrast, chronic PTU treatment induces a 3–4 fold increase in thyroid weight of normal controls whereas only a slight increase in thyroid weight results from this treatment in mice with transplanted pituitaries.

Although such results were believed best explained by postulating separate "growth" and "metabolic" thyrotropins, the growth factor requiring direct contiguity of the pituitary with the hypothalamus to be elaborated adequately, other explanations were possible (11). The present study was undertaken to see if there might be a difference in the sensitivity of the growth and metabolic responses of the mouse thyroid to both exogenous and endogenous thyrotropin. If the radioiodine uptake were found to have a significantly lower threshold, this would obviate the necessity of assuming the existence of more than one TSH.

MATERIALS AND METHODS

Female Swiss albino mice weighing approximately 20 gm. were used in all experiments. In studying the effect of exogenous thyrotropin, the animals were hypophysectomized by the parapharyngeal route one week prior to beginning the TSH injections. Parke, Davis thyrotropin (lot 50-P4), containing approximately 2 U.S.P. units per mg., was used in all experiments where exogenous TSH was given. Each dose was dissolved in 0.4 ml. physiologic saline and given subcutaneously once daily at rotated sites. To study endogenous thyrotropin secretion, animals were fed a diet containing 0.15% propylthiouracil for three weeks and injected intraperitoneally once daily with graded doses of sodium L-thyroxine dissolved in 0.2 ml. saline. Propylthiouracil was removed from the diet two days before autopsy to allow excretion of the drug. A Remington low iodine diet was fed except where otherwise indicated.

The animals were killed by exsanguination $4\frac{1}{2}$ hours following the intraperitoneal administration of 0.2 μC. I^{131} in 0.2 ml. physiologic saline. The thyroids were removed under

TABLE 1. HYPOPHYSECTOMIZED MICE GIVEN EXOGENOUS TSH

Exp.	Dose of TSH (mg.)	No. of animals	Body weight (gm.)	Thyroid weight		4½ hr. I^{131} uptake		% uptake/mg. thyroid
				(mg.)	% normal controls	% dose	% normal controls	
I	5	2	19.5±2.0	4.11±1.81	138.8±61.1	32.8±6.1	195.2±36.30	9.1±2.5
	2	3	19.3±1.0	4.28±1.00	144.7±33.8	34.2±1.4	203.4± 8.10	8.6±1.7
	0.5	5	19.5±0.6	3.10±0.36	104.6±12.2	21.4±4.8	127.5±28.70	6.8±1.5
	0.2	3	19.3±0.3	2.28±0.31	76.8±10.9	8.6±2.9	51.0±17.50	3.6±0.9
	0	4	18.0±1.5	1.78±0.27	60.2± 9.1	0.4±0.1	2.4± 0.50	0.2±0.0
	Normal Controls	7	25.0±1.5	2.96±0.34	100.0±10.0	16.8±1.1	100.0± 1.40	6.2±1.3
II	5	4	17.3±0.5	3.33±0.80	142.4±34.3	42.8±6.3	147.8±21.60	13.7±2.0
	2	5	17.9±0.8	2.10±0.24	89.7±10.3	21.4±4.9	73.9±16.80	10.2±1.9
	0.5	8	17.9±0.5	2.09±0.12	89.4± 5.1	21.9±1.9	75.5± 6.40	10.5±0.7
	0.2	7	17.5±0.5	1.64±0.09	69.9± 3.8	14.9±4.9	50.9±16.60	8.6±2.2
	0	4	16.3±0.6	1.92±0.26	82.1±11.0	0.5±0.1	1.5± 0.23	0.3±0.1
	Normal Controls	6	21.6±0.5	2.34±0.28	100.0±12.0	29.0±2.6	100.0± 8.90	13.6±2.5

Results expressed as mean and standard error. Indicated dose of TSH given once daily for 7 days, animals autopsied on 8th day.

a 6× dissecting scope and weighed on a Sartorious torsion microbalance with a sensitivity of 0.01 mg. Following weighing, the glands were placed in test tubes containing 2 ml. of water and counted in a scintillation well counter having a sensitivity of 10^6 cpm/μC. Completeness of hypophysectomy was checked by examination of the sella under 10× magnification at autopsy. Any animals with questionable pituitary remnants were discarded.

RESULTS

A. *Exogenous Thyrotropin:* The results are summarized in Table 1 and Figure 1. As has often been noted previously, hypophysectomy resulted in a much greater decline in radioiodine uptake than in thyroid weight. Unfortunately, because of our limited supply of thyrotropin and the insensi-

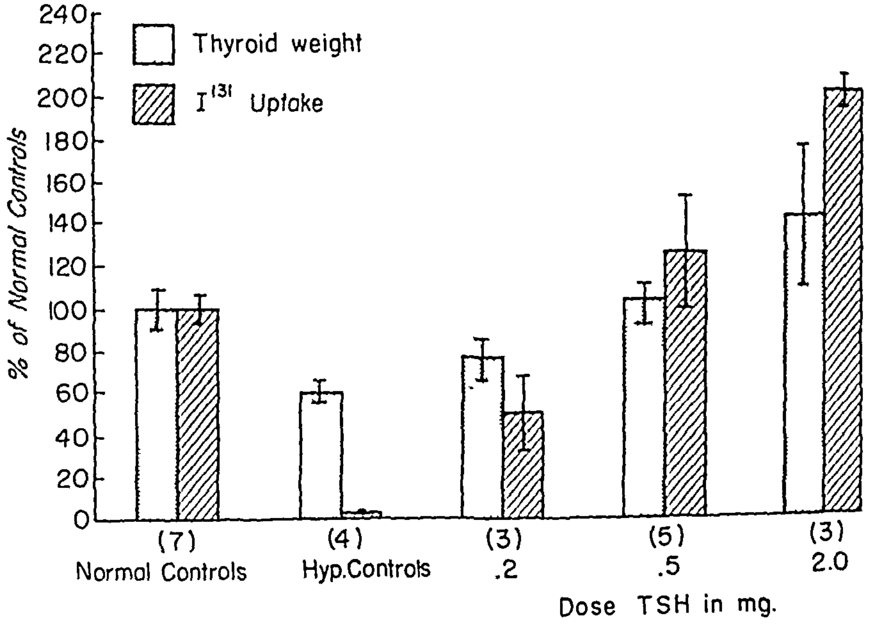

FIG. 1. TSH given to hypophysectomized mice. Graphic plot of data in Experiment I. Figures in parentheses refer to number of animals. Since only 2 mice survived in the group receiving 5 mg. TSH, this group was not plotted.

tivity of the thyroidal response, it was not possible to obtain a maximal effect from even the largest dose of thyrotropin given. Even with the submaximal response, however, it is apparent that a steeper dose-response slope was obtained with radioiodine uptake than with thyroid weight.

B. *Endogenous Thyrotropin:* The results are summarized in Table 2 and Figure 2. By using varying doses of thyroxine in combination with amounts of propylthiouracil which would assure as complete inhibition of thyroxine synthesis as could be obtained, it was possible to control the rate of secretion of endogenous thyrotropin over as wide a range as desired. The steeper dose-response slope of radioiodine uptake obtained with exogenous thyrotropin was confirmed with endogenous TSH. In addition, it was found that with the faster rate of increase of the radioiodine uptake this parameter reached a plateau with a dose of thyroxine at least 3 times as large as that used when a plateau in thyroid weight was reached. This indicated that a *much smaller amount of circulating thyrotropin caused maximal radioiodine uptake than was required to produce maximal thyroid weight.*

It is of interest that an "overshoot" was produced at the beginning of the radioiodine uptake plateau (Fig. 2). Once the dose of thyroxine had been reduced to a point where the uptake had increased sufficiently to equal that of the PTU controls, a further reduction in thyroxine dosage would consistently result in an increase in radioiodine uptake above that seen in the PTU controls. As the dose of thyroxine was reduced to zero, the

TABLE 2 PTU-FED MICE GIVEN GRADED DOSES OF THYROXINE

Exp	Dose of thyroxin	No of animals	Body weight (mg)	Thyroid weight		4½ hr I^{131} uptake		% uptake/mg thyroid
				(mg)	% PTU cont	% Dose	% PTU cont	
III	10	7	20.6±1.72	1.83±0.11	20.0± 1.20	0.47±0.09	1.7± 0.50	0.26±0.08
	5	7	20.9±1.29	2.17±0.20	23.7± 2.20	0.50±0.04	1.8± 0.15	0.24±0.03
	2	7	21.9±0.86	2.39±0.51	27.7± 5.30	9.40±4.80	32.9±16.70	2.87±0.93
	0.5	7	21.6±1.28	7.00±0.98	76.2±10.70	35.60±4.90	124.5±16.90	6.28±1.71
	0	7	22.4±0.71	9.15±0.69	100.0± 0.13	28.60±1.56	100.0± 0.42	3.24±0.29
	Purina Controls	5	24.9±1.70	1.98±0.13	21.6± 1.40	2.20±0.42	7.7± 1.40	1.12±0.23
	L I Controls	6	21.7±1.33	2.99±0.88	32.7± 9.60	23.40±5.50	81.9±19.20	10.43±3.85
IV	5	8	24.0±0.44	2.43±0.11	21.5± 1.00	3.90±0.90	6.4± 1.50	1.60±0.31
	2	8	21.3±1.00	4.53±0.74	40.1± 6.50	38.30±8.20	62.7±13.40	8.45±2.40
	1	7	21.6±0.79	10.50±0.62	92.8± 5.50	85.70±1.10	140.3± 1.70	8.16±0.48
	0.7	8	22.6±0.69	11.92±0.87	105.4± 7.70	82.50±2.40	135.0± 4.00	6.92±0.66
	0.5	8	21.0±0.81	12.59±1.40	111.4±12.50	65.80±4.60	107.7± 7.50	5.23±0.45
	0.2	8	20.8±0.50	10.70±0.97	94.6± 8.60	61.50±5.40	100.7± 8.80	6.26±1.23
	0	8	20.9±0.56	11.31±1.10	100.0± 0.83	61.10±2.10	100.0± 2.13	5.40±0.32
	L I Controls	8	22.8±0.81	3.57±0.30	31.6± 0.50	53.90±2.80	88.2± 4.60	15.10±1.27
V	5	9	19.2±0.38	1.83±0.10	23.2± 1.30	2.40±0.40	4.3± 0.80	01.40±0.30
	3	11	19.4±0.50	2.38±0.37	30.2± 4.70	15.80±4.50	28.0± 7.90	05.60±1.20
	2	11	20.0±0.33	3.34±0.28	42.5± 3.40	49.80±6.40	88.1±10.40	13.80±1.60
	1.5	10	17.9±0.75	4.27±0.64	54.2± 8.10	61.20±8.70	108.5±13.60	15.30±2.60
	1	10	18.3±0.60	4.84±0.46	61.5± 5.90	63.50±6.40	112.6±11.00	13.50±1.30
	0.7	7	20.7±0.50	5.81±0.65	73.8± 8.30	63.80±5.70	113.1±10.20	09.10±0.80
	0.5	9	19.3±0.50	7.21±0.64	91.6±10.00	58.80±3.50	104.3± 6.30	09.00±1.30
	0	8	19.1±0.68	7.87±0.69	100.0± 8.80	56.40±2.70	100.0± 4.80	07.80±0.70

Results expressed as mean and standard error. Indicated dose of thyroxine given daily. Purina controls were fed Purina fox checkers. All groups were fed propylthiouracil in the diet except the Purina controls and low iodine controls. All animals except the Purina controls were fed a low iodine diet. PTU Cont =animals fed the low iodine PTU diet but did not receive thyroxine. L I Controls =animals fed low iodine diet. Neither the Purina nor low iodine control groups received thyroxine.

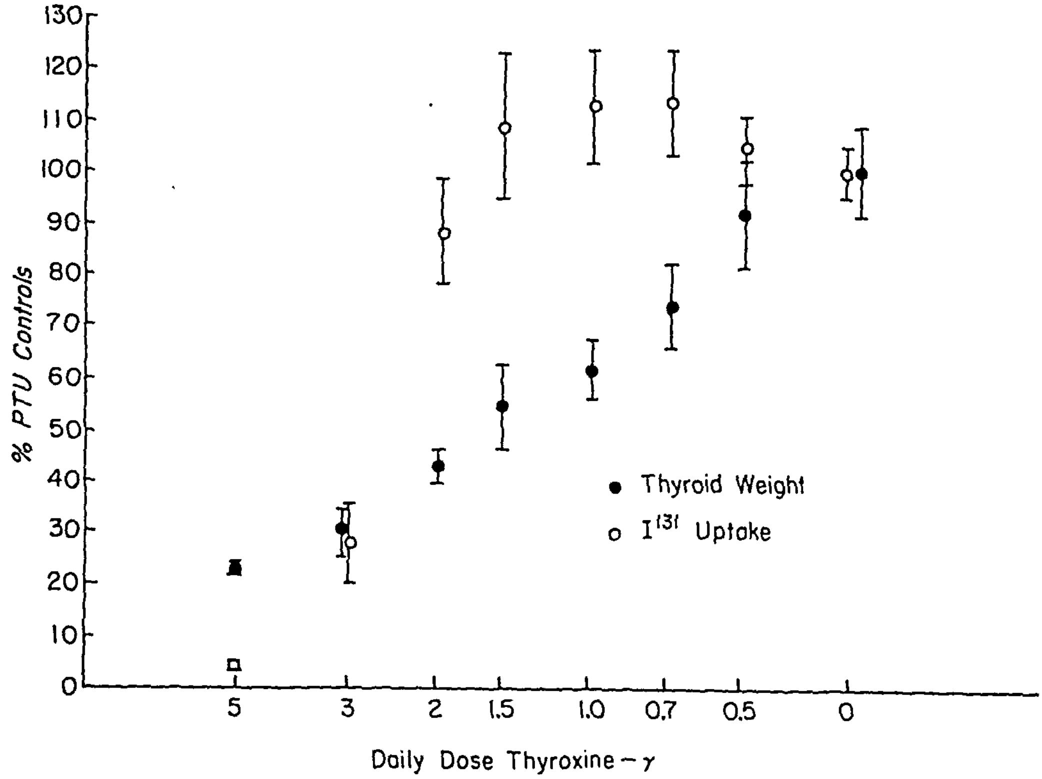

FIG. 2. Thyroid weight vs. uptake in PTU-fed mice administered thyroxine. Graphic plot of data in Experiment V.

iodine uptake would fall to the same level observed in the PTU controls. The height of the overshoot was maximal at a daily dose of 0.7–1.0 μg. thyroxine.

DISCUSSION

The results obtained in these experiments support the concept that the high radioiodine uptake with little thyroid growth response exhibited by mice with heterotopic pituitaries may be due to a greater sensitivity of thyroidal radioiodine metabolism than thyroid growth to small amounts of thyrotropin. If both growth and iodine metabolism were equally sensitive, one would expect that both parameters would reach a plateau at the same level of thyrotropin. If one were more sensitive, its plateau would be expected to begin at a smaller dose. The data recorded here clearly indicate that a plateau was first reached with radioiodine uptake.

In some of the earlier experiments in mice with heterotopic pituitaries (5), chronic propylthiouracil treatment produced an absolute increase in the radioiodine uptake per unit thyroid weight of such animals which was 4-fold greater than that produced in similarly treated normal controls. Failure to achieve this level in the present experiment is more likely due to to individual variations in the animals than to a qualitative difference in the response. Not all groups of mice with pituitary transplants previously studied (5) achieved a 4-fold greater uptake per unit thyroid weight than their controls. It is of interest that in the present experiments the highest I^{131} uptake/mg. thyroid occurred in animals receiving low iodine diet alone.

The "overshoot" of the radioiodine uptake with increasing amounts of endogenous TSH was seen consistently in all experiments, although its significance is not clear at this time. It possibly suggests further the negative feedback control system by which the level of circulating thyroxine regulates TSH output, overshoots of this nature being characteristic of "servo" mechanisms. Another possible explanation is that a small amount of thyroxine may enable the pituitary to secrete more TSH than it does in the complete absence of thyroxine. Such a concept is supported by previous evidence in human myxedema where the urinary or blood levels of TSH are transiently increased after the initiation of therapy with thyroid hormone (9, 10).

Our interpretation of the present results is that they add to a growing body of evidence suggesting that the apparent divergence of various parameters of thyroid function under certain conditions may more likely be explained by different thresholds of the individual processes to a single thyrotropin than that more than one thyrotropin is involved in their control. It is worth noting, however, that Florsheim (8) has recently found that thyroidal radioiodine uptake and protein-bound iodine can be suppressed in the rat by hypothalamic lesions which do not prevent thyroid hypertrophy due to administration of antithyroid drugs. Since this is the converse of the present findings, the possible existence of more than one thyrotropin has not yet been finally excluded.

Acknowledgment

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FUNCTIONAL ZONATION OF THE ADRENAL CORTEX: PATHWAYS OF CORTICOSTEROID BIOGENESIS

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ABSTRACT

Direct proof that aldosterone is produced by the glomerulosa of beef and rat adrenals has recently been provided. In the present investigation, the functional zonation of beef adrenal cortex has been further studied *in vitro*.

When progesterone, desoxycorticosterone and corticosterone are added to the incubation media of beef adrenal slices they are metabolized in radically different ways depending on whether the slices originate in the glomerulosa (G) or in the fasciculata-reticularis (F.R.). In the presence of tissue taken from G these steroids increase the production of aldosterone.

Desoxycorticosterone increases the formation of corticosterone both in G and F.R. Progesterone enhances the production of hydrocortisone in F.R. only, whereas it increases corticosterone and aldosterone production in G. 21-hydroxypregnenolone increases the production of aldosterone in G and that of corticosterone in both G and F.R. 17α-hydroxyprogesterone and 17α-hydroxy-11-desoxycorticosterone markedly enhance the production of hydrocortisone in F.R. and lead to the formation of this compound in G where normally it is not formed. Progesterone, desoxycorticosterone and corticosterone increase the production of aldosterone by G to the same extent.

These findings provide indirect evidence for an enzymatic specificity within the different types of cortical cells which is responsible for the anabolism of different types of hormones from a possibly identical precursor. Thus, the evidence tends to indicate the occurrence of 18-oxygenation in G but not in F.R., of 17-hydroxylase activity in F.R. but not in G, and that of 3β-dehydrogenase activity and 11- and 21-hydroxylase activities in both.

INTEREST in the functional specificity of the different cellular zones of of the mammalian adrenal cortex arose from the inspiring investigations of Deane, Greep and collaborators (1, 2, 3) who first correlated morphological changes of the adrenal cortex with changes in mineral balance, pituitary function, and carbohydrate metabolism.

Although their conclusions were confirmed by a number of workers the validity of their methods, as well as the interpretation of their results, has been debated. Following extensive studies of mice adrenal cortex, Chester Jones (4) concluded that in eutherian mammals the existence of the glomerulosa is only due to the presence of a poorly elastic capsule of connective tissue and that this zone represents a "vegetative back-water" of cortical

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cells. However, the recent demonstration that aldosterone is exclusively produced by the zona glomerulosa of beef and rat adrenals (5–8) has lent great support to the conclusion of Deane, Greep and their group.

The aim of the following investigation is to provide further evidence for the concept of "functional zonation" by demonstrating the existence of different pathways of corticosteroid biogenesis within the different cellular layers of the adrenal cortex.

METHODS

Incubation

Beef adrenal glands were collected at the slaughterhouse within the hour following the death of the animals. The glands were dissected free of peripheral fat and bisected. The medulla was discarded. The cortex, with the remaining connective tissue of the capsule, was cut into slices 1–1.5 cm. wide and 0.3–0.5 mm. thick. The zona glomerulosa was separated from the two inner cortical zones by cutting with a razor blade along a line of pigmented cells, visible to the naked eye, which was histologically proven to correspond with the innermost cellular layers of the glomerulosa (see Figure 1). The slices were then transferred into the compartments of a partitioned Petri dish, in a solution of 0.9% sodium chloride. In each series of experiments, the glomerulosa slices and their fasciculata-reticularis counterparts were evenly distributed among the different groups of tissue to be incubated so that equally representative parts of the cortex could contribute to each group of tissue. From the time of collection at the slaughterhouse until the beginning of the incubation, an average period of three hours, the tissues were kept in a solution of 0.9% sodium chloride at 5 to 8° C—except for the time necessary to prepare the slices. Prior to incubation, the slices were decanted, gently blotted on filter paper to absorb excess saline, and weighed on a torsion balance. Each group of slices was then transferred to a 50 cc. beaker containing 10 cc. of incubation medium per gram of tissue. The slices were incubated in Krebs Ringer Bicarbonate medium with added glucose (200 mg.%) (9, 10). This medium was always freshly prepared from concentrated mother solutions kept at 5° C and renewed every 4 months. The incubation was performed in a Dubnoff Metabolic Shaker Incubator in an atmosphere of oxygen—5% CO_2, at 37 ± 1° C. The flow of gas and the shaking were regulated at 5 liters/minute, and 100 movements/minute, respectively. According to the method recommended by Saffran (10), the slices were pre-incubated for a period of half an hour, at the end of which the medium was renewed and the incubation (per se) allowed to proceed for 2 hours. When corticosteroids precursors were used, chosen amounts of these compounds, kept in methanol solution, were pipetted into a set of clean beakers. The methanol was evaporated off under a stream of nitrogen. The steroids were redissolved in the Krebs Ringer Bicarbonate medium which, if necessary, was slightly warmed to hasten the solubilization. The pre-incubated slices were then transferred to these beakers. At the end of the incubation, the media were collected and the slices rinsed a few times with small portions of distilled water. The rinsings were added to the respective incubation media. These were frozen until extraction. In most experiments 1.5–2.0 gm. of glomerulosa and 3.0–4.0 gm. of fasciculata-reticularis were used to make up individual groups of slices but up to three times these amounts were needed when the incubation was performed in the absence of added steroid precursors.

Extraction

The media were extracted first with twice, and then with once their volume of chilled, redistilled chloroform. Emulsions were broken down by centrifugation. The chloroform extracts were pooled, filtered into round-bottom flasks so as to absorb residual traces of water, and evaporated to 2 cc. under vacuum at a water bath temperature of 50 ± 2° C. The concentrated extract was dried under nitrogen. In each set of experiments an appropriate volume of incubation medium was extracted to be used eventually as a "blank."

Crude Extracts

In a number of experiments, aliquots of the crude lipid extracts were taken for quantitative analysis of total Δ^4-3 ketonic steroids at 2400 Å (10), of total α-ketolic reducing steroids by the micro blue tetrazolium reaction of Genest (11) and of dihydroxyacetone steroids by the Porter-Silber reaction (12). These determinations were performed against proper aliquots of the incubation medium "blank" carried through the entire procedure. In most cases 1/20 of the crude lipid extract of each incubation medium was sufficient for each of these determinations.

Chromatography

All chromatographic developments were done at room temperature (25 ± 2° C), after overnight equilibration for the system of Bush (13), and after 12 hours equilibration at 35° C, in a constant temperature cabinet for the system E^2B of Eberlein Bongiovanni (14). Detection of the steroids on the chromatograms was achieved by direct visualization and/or contact photography under ultraviolet light. If elution of the steroids was not performed, the combined blue or triphenyltetrazolium NaOH fluorescence reaction (15) was developed on the paper. The crude lipid extracts were in all cases submitted to a first chromatography in the system B5 of Bush (benzene-methanol-water, 2:1:1) over a period of 4 to 5.5 hours.

Partial Identification

Partial identification of aldosterone, hydrocortisone, and corticosterone was achieved by assessing their respective chromatographic mobilities in the system propyleneglycol: toluene of Zaffaroni (16), in the system C of Bush and in the system E^2B of Eberlein Bongiovanni; by mixed chromatography with the corresponding crystalline compound and by the identity of their respective sulfuric acid spectra with that of pure compounds (15). Aldosterone was furthermore characterized by its biological activity in either the Venning (17) or the Simpson-Tait (18) assay. Aldosterone, hydrocortisone and corticosterone were subjected at least once to this complete series of tests in each group of experiments, but not in individual experiments where the conditions of incubation and the precursor studied remained unchanged.

Spectrophotometric Determination

The quantitative determination of Δ^4-3 ketosteroids was carried out at 2400 Å according to the method described by Saffran (10). Spectrophotometric measurements of these steroids were performed in methanol solution against the eluate of a similar area of the paper on which the extract of the incubation medium "blank" had been chromatographed. Hydrocortisone and corticosterone were quantitatively measured by this method after one single chromatography in the system B5; aldosterone after a second chromatography in the system C of Bush. A greater chromatographic purification of aldosterone was not considered essential for the following reasons:

1) after two successive chromatographic developments in the systems B5 and C aldosterone if reapplied on the iso-octane tert-butanol aqueous methanol system (14) behaves as homogeneous Δ^4-3 ketone material.
2) the values of aldosterone obtained by this method are very close to those obtained by bioassay.

RESULTS

In a preliminary experiment an attempt was made to incubate separately the three zones of beef adrenal cortex. Consequently, after the separation of the zona glomerulosa the innermost fourth of the fasciculata-reticularis slices was separated. This innermost portion of the cortex is designated here zona reticularis. Although in beef adrenal cortex the existence of a well-defined zona reticularis is not unanimously recognized, the concept of certain authors considering the innermost part of the cortex to be a zone of cellular degenerescence made it of interest to investigate its ability to produce hormones upon incubation. The quantitative estimation of Δ^4-3 ketosteroids, α-ketolic and 17-hydroxycorticosteroids produced on incubation by the glomerulosa, the fasciculata, and the reticularis of beef adrenal cortex is presented in Table 1 together with the total sodium-retaining activity of the crude lipid extracts of their respective incubation media

From this experiment it appeared that 17-hydroxycorticosteroids (Porter-Silber chromogens) are produced almost entirely by the fasciculata and the reticularis. Assuming that the beef adrenal cortex produces negligible amounts of 17-hydroxycorticosteroids reduced in ring A, one is led to conclude that by far the largest portion of Δ^4-3 ketosteroids produced by the fasciculata and the reticularis bears a dihydroxyacetone side chain, whereas Δ^4-3 ketosteroids bearing an α-ketolic side chain originate mostly in the glomerulosa. The ratio of Porter-Silber chromogens/Δ^4-3 ketoster-

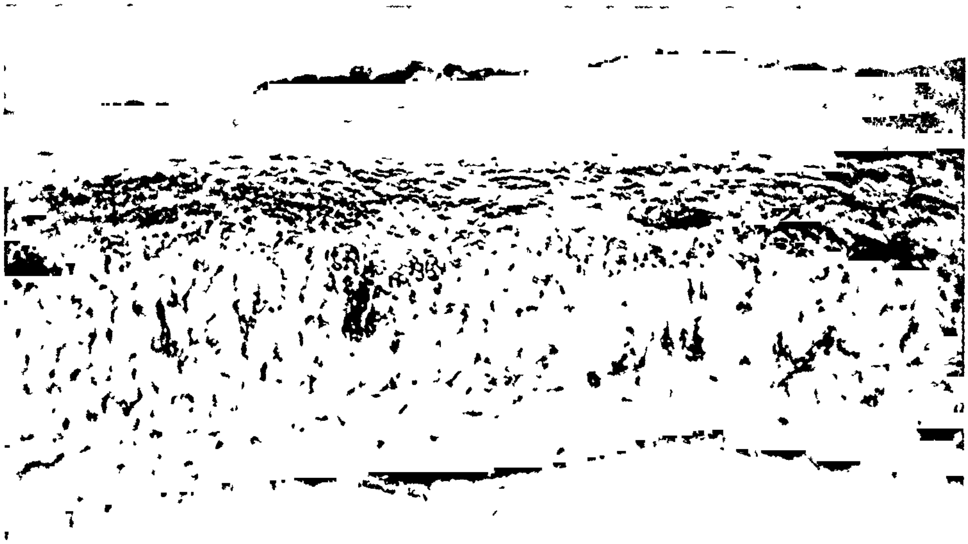

FIG. 1. Microphotograph of glomerulosa slice (×85; Masson Trichrome). Connective tissue of the capsule is seen adhering to zona glomerulosa.

TABLE 1. COMPARISON OF THE STEROID MATERIAL PRODUCED ON INCUBATION BY THE DIFFERENT CELLULAR ZONES OF BEEF ADRENAL CORTEX

	μg./100 gm. of tissue/hour			
	U.V.[1]	B.T.Z.[2]	P.S.[3]	M.A.[4]
Glomerulosa	1030	750	20	130
Fasciculata	450	375	270	4
Reticularis	400	270	180	0

[1] Maximal absorption at 2400 Å.

[2] Blue Tetrazolium reaction.

[3] Porter-Silber reaction.

[4] Mineral-corticoid activity in the Simpson-Taits assay (18) expressed in μg. of aldosterone.

oids is equal to .02 in the glomerulosa as opposed to 0.6 and 0.45 in the fasciculata and the reticularis respectively.

The table also shows that a certain proportion of steroids possessing a Δ^4-3 ketone group but no 17-α-ketolic side chain is produced in the three cortical zones, possibly in slightly greater amounts by the zona glomerulosa.

Finally, it is most striking to note that practically all the sodium-retaining activity is found in the crude extract of the incubation medium of glomerulosa slices, whereas it is negligible in the incubation media of the fasciculata and the reticularis. Following this experiment, it was not again attempted to incubate the zona reticularis separately.

After paper chromatography, the main compounds produced by incubation of glomerulosa slices were identified as aldosterone and corticosterone; those produced by the zona fasciculata-reticularis as hydrocortisone and corticosterone (see Figure 2).

No aldosterone was detected on the chromatograms of the incubation media of fasciculata-reticularis, and only traces of hydrocortisone on the chromatograms of the incubation media of glomerulosa. It is highly probable that these traces of hydrocortisone, which in all cases were too small to be measured by spectrophotometry, were produced by clusters of fasciculata cells incompletely separated from the zona glomerulosa during dissection of the tissue.

Two unidentified ultraviolet absorbing substances which did not reduce tetrazolium salts were found to be regularly produced in the incubation media of glomerulosa slices. In the system B5 of Bush their chromatographic mobilities were respectively slower than that of the hydrocortisone pilot and slightly faster than that of the cortisone pilot. Besides hydrocortisone and corticosterone two Δ^4-3 ketone, α-ketolic compounds were consistently observed after chromatography of the extracts of the incubation media of fasciculata-reticularis slices. In the system B5 of Bush, one was more polar than hydrocortisone, the second had a polarity very similar to the pilot of cortisone. These substances are not homogenous and can be

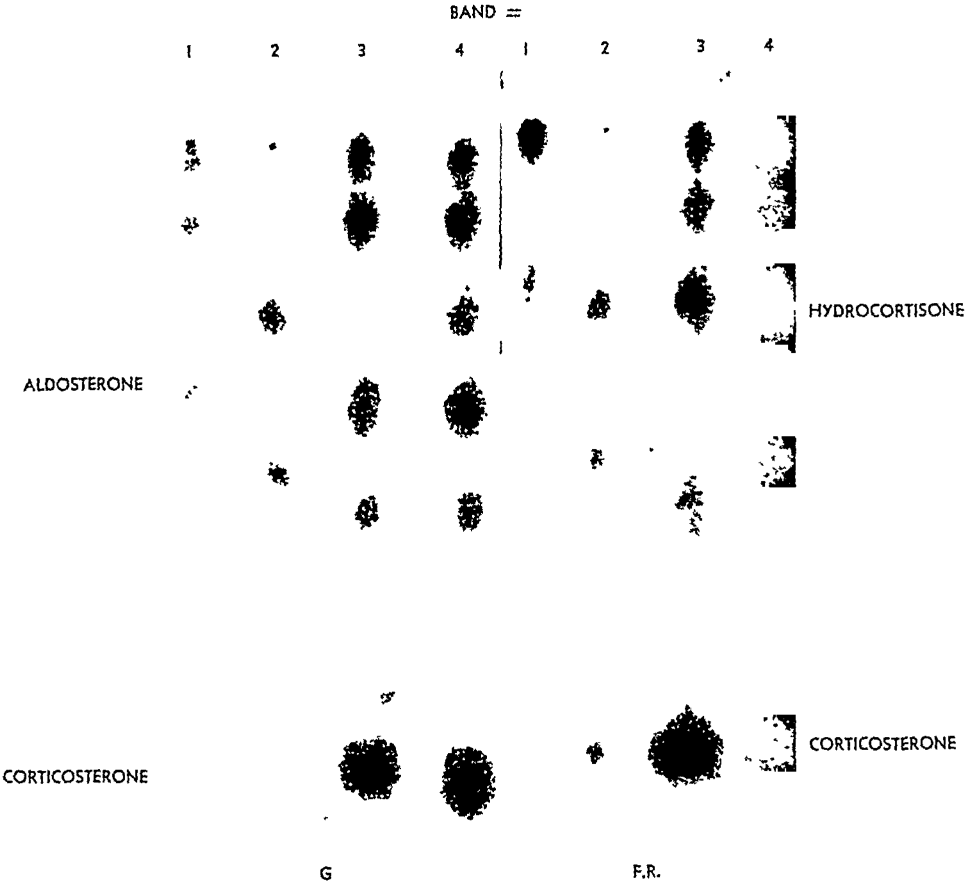

FIG. 2. U.V. photostat of chromatograms from the system B5 of Bush on which extracts of incubation media of glomerulosa (G) and fasciculata-reticularis (F.R.) slices were developed. Band 1: "Control"; Band 2: Pilots Hydrocortisone, Cortisone and Corticosterone: 10 μg. of each; Band 3: incubation with desoxycorticosterone; Band 4: incubation with progesterone.

resolved into two or three compounds by further chromatography. Finally, a steroid, partly identified as 11-dehydrocorticosterone, was recognized as being produced by both parts of the cortex.

The lowest part of Table 2 shows that the basic production of aldosterone by glomerulosa slices is equal to 37 ± 5 μg./100 gm. of tissue/hour; that of hydrocortisone by the fasciculata-reticularis to 61 ± 6 μg./100 gm. of tissue/hour. Corticosterone is produced at the rate of 58 ± 8 and 45 ± 9 μg./100 gm. of tissue/hour by glomerulosa and fasciculata-reticularis slices, respectively. When known precursors of corticosteroids such as desoxycorticosterone, progesterone, and corticosterone were added to the incubation media at the dose level of 50 μg./gm. of tissue/2 hours of incubation, they were handled in radically different ways depending on whether

TABLE 2. PRODUCTION OF ALDOSTERONE, HYDROCORTISONE AND CORTICOSTERONE BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH DESOXYCORTICOSTERONE (DOC), PROGESTERONE AND CORTICOSTERONE (B)

Steroid added[1]	Glomerulosa				Fasciculata-Reticularis			
	Tissue weight incubated (mg.)	μg./100 gm. tissue/hour			Tissue weight incubated (mg.)	μg./100 gm. tissue/hour		
		Aldosterone	Hydrocortisone	Corticosterone		Aldosterone	Hydrocortisone	Corticosterone
DOC	2372	82	traces	290	3709	Nil	150	350
	1824	109	traces	576	3360	Nil	30	120
	1898	75	traces	130	2852	Nil	78	280
	1892	127	traces	500	2882	Nil	70	340
Progesterone	2355	79	traces	250	3615	Nil	380	140
	1985	115	traces	75	3898	Nil	320	60
	1761	134	traces	298	3210	Nil	155	62
	1962	133	traces	490	3010	Nil	255	180
B	1795	115	traces	Not measured	3486	Nil	60	Not measured
	1674	220	traces	Not measured	2944	Nil	60	Not measured
	1976	142	traces	Not measured	3900	Nil	59	Not measured
Mean Control production μg./100 gm. tissue/hr.		37 ± 5*	traces	58 ± 8		Nil	61 ± 6	45 ± 9

[1] 5 μg./100 mg. of tissue/2 hours of incubation.
* Standard Error of the mean.

the slices originated in the glomerulosa or in the fasciculata-reticularis (see Table 2 and Figure 2). In the presence of glomerulosa slices, these three steroids increased the production of aldosterone, but they did not lead to the formation of this hormone by the fasciculata-reticularis. Only progesterone enhanced the production of hydrocortisone, and this only in the incubation media of fasciculata-reticularis slices. The addition of progesterone, desoxycorticosterone, and corticosterone to the incubation media of glomerulosa slices did not promote the formation of hydrocortisone which, as in the control, was found only in negligible amounts. However, if important fragments of fasciculata remained attached to the glomerulosa, measurable amounts of hydrocortisone were found in the incubation media of glomerulosa slices after incubation with progesterone.

In both the glomerulosa and the fasciculata-reticularis, desoxycorticosterone markedly increased the production of corticosterone; whereas progesterone enhanced the production of this steroid mainly in the zona glomerulosa. On the other hand, 17-hydroxylated steroid precursors such as 17-hydroxyprogesterone and 17-hydroxy-11-desoxycorticosterone led to the formation of large amounts of hydrocortisone, not only in the fasciculata-reticularis, but also in the glomerulosa (see Table 3).

It can be calculated that in the presence of glomerulosa slices, these steroids have been converted to hydrocortisone to an extent of 60%. This observation demonstrates the efficiency of 21- and 11-hydroxylations on a 17-hydroxylated precursor incubated with glomerulosa tissue.

In order to study the capacity of the glomerulosa and the fasciculata-reticularis to promote the transformation of the Δ^5-3 ol to the Δ^4-3 ketone structure, it was decided to incubate pregnenolone in the presence of these two parts of the cortex. However, since pregnenolone was not available, its

TABLE 3. PRODUCTION OF HYDROCORTISONE (F) BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH 11-DEHYDRO-17-HYDROXY-CORTICOSTERONE (S) AND 17-HYDROXY-PROGESTERONE (17-OH-PROG.)

Steroid added µg./100 mg. of tissue	Tissue weight incubated (mg.)	Glomerulosa (F: µg./100 gm. tissue/hr.)	Tissue weight incubated (mg.)	Fasciculata-reticularis (F: µg./100 gm. tissue/hr.)
S: 10 µg.	1540	1460	3204	1120
S: 10 µg.	1540	1900	3204	1030
S: 10 µg.	1425	1400	1680	1710
S: 10 µg.	1425	1770	1680	2900
17-OH-Prog. 5 µg.	1518	490	1835	810
17-OH-Prog. 5 µg.	1518	540	1835	580
17-OH-Prog. 10 µg.	1500	2100	3176	780
17-OH-Prog. 10 µg.	1500	2670	3176	590
Mean Production of Hydrocortisone by Controls µg./100 gm./hour		traces		55 ± 3.8[1]

[1] Standard Error of the mean: 8 experiments.

21-hydroxylated derivative (at the dose of 100 µg./gm. of tissue/4 hours of incubation) was used in the experiment reported in Table 4. 21-hydroxypregnenolone increased the production of aldosterone by the zona glomerulosa and markedly enhanced the production of corticosterone by both parts of the cortex. The production of hydrocortisone by the fasciculata-reticularis was also increased but to a lesser extent. This may reflect a limited ability of 17-hydroxylation on a 21-hydroxylated substrate.

To investigate further the pathway of aldosterone biogenesis, glomerulosa slices were incubated in the presence of increasing amounts of progesterone, desoxycorticosterone and corticosterone. Each of these steroids was added to the incubation media at the dose levels of 5, 10 and 20 mg. /100 gm. of glomerulosa tissue. The period of incubation lasted 2 hours. The results of these experiments are summarized in Figure 3. They show that the production of aldosterone/unit of tissue weight/hour was proportional to the amount of steroid added, and that at a given dose these three steroids increased the production of aldosterone above that of the controls in

TABLE 4. PRODUCTION OF ALDOSTERONE, HYDROCORTISONE AND CORTICOSTERONE BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH 21-HYDROXYPREGNENOLONE

Steroid added[1]	Glomerulosa				Fasciculata-Reticularis			
	Tissue weight incubated (mg.)	µg./100 gm. tissue/hour			Tissue weight incubated (mg.)	µg./100 gm. tissue/hour		
		Aldosterone	Hydrocortisone	Corticosterone		Aldosterone	Hydrocortisone	Corticosterone
21-OH Pregnenolone	1320	240	traces	393	2468	Nil	210	250
	1338	299	traces	239	2608	Nil	144	138
	1356	310	traces	200	2395	Nil	117	200
Control #1	1600	99	Nil	18	3086	Nil	47	18
Control #2	1500	86	Nil	26	2614	Nil	55	16

[1] 10 µg. of 21-OH-Pregnenolone/100 mg. of tissue/4 hours of incubation.

strictly comparable proportions. For instance the amounts of aldosterone recovered after incubation with progesterone, desoxycorticosterone and corticosterone were respectively equal to 250 ± 37 (S.D.), 253 ± 39, and 250 ± 40 μg./100 gm. of tissue/hour when these steroids were added to the incubation media at the dose of 10 mg./100 gm. of glomerulosa slices. These figures represent a mean increase of almost 600% above the mean production of the controls (37 ± 10), and a conversion of 5% of the added steroids to aldosterone over the 2 hours of incubation.

Regarding the 18-oxygenation occurring only in the glomerulosa it may be of interest to mention the following observation. When 11-dehydrocorticosterone and 11 α-hydroxyprogesterone were incubated with the glomerulosa, two compounds were obtained possessing a chromatographic mobility practically identical with that of aldosterone in the systems B5 and C of Bush and E^2B of Eberlein-Bongiovanni. These two compounds were shown to possess a maximal absorption at 2400 Å in methanol solution and to give a typically positive soda fluorescence reaction; but they neither reduced blue tetrazolium salt nor did they present sodium-retaining activity on bioassay. Since these compounds were essentially formed in the glomerulosa, it is tempting to credit them both with an oxygen function on position 18. One could therefore speculate that 11-dehydrocorticosterone would give rise to 11-dehydrocorticosterone 18-al; 11 α-hydroxyprogester-

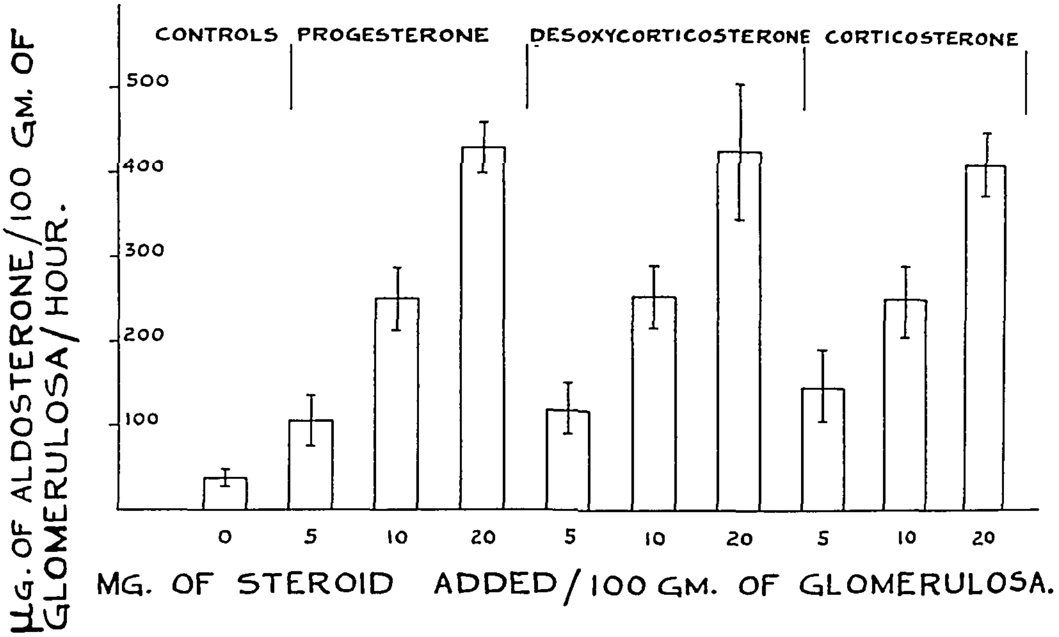

FIG. 3. Production of aldosterone by the zona glomerulosa of beef adrenal incubated with progesterone, desoxycorticosterone and corticosterone ordinate is μg. of aldosterone produced on incubation per 100 gm. of glomerulosa/hour when the tissue was incubated in the presence of increasing doses of progesterone, desoxycorticosterone and corticosterone. The vertical lines represent the magnitude of the standard deviations of the mean.

one to 11-epialdosterone or to 11-dehydrocorticosterone 18-al. The preferential formation of 11-dehydrocorticosterone 18-al could find support in the finding of large amounts of 11-dehydrocorticosterone in the incubation media of glomerulosa slices incubated in the presence of 11α-hydroxyprogesterone in accordance with the original finding of Zaffaroni (19) in beef adrenal homogenates. In either case the formation of aldosterone hemiacetal not being possible, there could be interaction between the 18-aldehyde and the α-ketolic side chain which would explain the lack of reducing properties of the latter.

DISCUSSION

The concept of the functional zonation of the adrenal cortex arose primarily from Swan's hypothesis (20) which implies that the zona glomerulosa is responsible for the regulation of electrolyte metabolism and is little affected by hypophysectomy whereas the fasciculata-reticularis, highly dependent on the presence of the anterior pituitary, is the site of formation of carbohydrate-regulating hormones. This view was, for the first time, put to the experimental test by Sarason (21) who demonstrated a regression of the zona glomerulosa in rats receiving desoxycorticosterone and later by Deane, Greep and their collaborators who fully confirmed and extended this experimental finding by correlating histochemical changes in the glomerulosa and the fasciculata-reticularis of rat adrenals with induced changes in carbohydrate and electrolyte metabolism (1, 2, 3).

The recognition of aldosterone as the chief mineralocorticoid evoked the interest of various investigators in the possible site of its production within the adrenal cortex, until different groups of workers provided direct proof of its elective production by the zona glomerulosa (5, 6, 7, 8).

The present experiments confirm the original findings of Ayres, Gould, Simpson, and Tait on the production of aldosterone by the glomerulosa, of hydrocortisone by the fasciculata, and of corticosterone by both layers of the ox adrenal gland (5). They indicate further that practically all the biologically active substances controlling electrolyte metabolism (sodium-retaining substances) are produced by the zona glomerulosa, but do not support the conception that the innermost cellular layers of the cortex are formed of degenerating or dying cells. This last assertion is based on the observation that, on incubation, cortical slices from the innermost layer of the cortex produced steroid materials similar to those produced by the fasciculata.

The present investigation provides strong evidence for the existence of different pathways of corticosteroid biogenesis within different parts of the cortex. Its significance lies mostly in the observation that in the presence of an identical steroid precursor, a definite kind of cell is specifically and exclusively able to achieve the whole metabolic transformation necessary for the formation of specific hormones recognized in a number of animal species, including man, as being the chief products of adrenocortical secretion. For example, progesterone, through 17, 21 and 11-hydroxylations (22), leads to the formation of hydrocortisone only by the fasciculata-reticularis and, through 21-, 11-hydroxylations, and 18-oxygenation, to the formation of aldosterone only by the glomerulosa. These findings imply the localization within specific areas of the cortex of some enzymes recently isolated from cortical tissue and shown to participate in corticoid biogenesis. They make it possible to outline an enzymic zonation of the adrenal cortex which would be characterized by the presence of both specific and common enzymic activities within the glomerulosa and the fasciculata-reticularis.

Thus, 17-hydroxylase activity would be found essentially in the cells of the fasciculata-reticularis, since progesterone increases the production of hydrocortisone in the presence of slices formed of this type of cells, but does not promote the formation of this steroid on incubation with glomerulosa cells. Conversely, 18-oxygenation would occur only in the cells of of the glomerulosa as indicated by the observation that progesterone, desoxycorticosterone, and corticosterone enhance the production of aldosterone by these cells but do not lead to its formation by fasciculata-reticularis cells. Finally, 3β-dehydrogenase activity and both 11- and 21-hydroxylase activities would be found throughout the cortex since 21-hydroxypregnenolone leads to an increase of aldosterone and hydrocortisone production in the glomerulosa and the fasciculata-reticularis respectively, and since the conversion of progesterone to hydrocortisone and aldosterone requires both 21- and 11-hydroxylations.

In recent years studies on the biogenesis of aldosterone have led to a number of conflicting data. In beef adrenal homogenates incubated with added glucose, fumarate, nicotinamide and ATP, Wettstein *et al.* have shown that the yield of aldosterone is increased by desoxycorticosterone but is unaffected or even decreased by progesterone and corticosterone. The authors concluded that their results indicated the occurrence of a sequence of hydroxylation in 21, 18, and 11 during the biogenesis of aldosterone. Kahnt reinforced this conclusion by demonstrating the formation of C^{14}-aldosterone upon incubation of C^{14}-desoxycorticosterone (23, 24).

At variance with these results are those of Rosemberg *et al.* (25) who showed that after perfusion of isolated calf adrenal with progesterone, desoxycorticosterone, and corticosterone, and subsequent biological measurement of the aldosterone fraction partially purified from the effluent, only progesterone resulted in a significant increase of the sodium-retaining activity. Corticosterone gave equivocal results and desoxycorticosterone was without action. These conclusions might receive some support from preliminary observations of small yields of C^{14}-aldosterone after perfusion of beef adrenal with C^{14}-progesterone (26). On the other hand, although Travis and Farrell showed that incubation of progesterone-4-C^{14} with beef adrenal capsule strippings resulted in the production of aldosterone-4-C^{14} of approximately the same molar activity, incubation with corticosterone-4-C^{14} yielded aldosterone possessing a molar specific activity of up to 54% of corticosterone-4-C^{14} (27).

In the present study, incubation of beef glomerulosa tissue in the presence of identical amounts of progesterone, desoxycorticosterone and corticosterone have been observed to increase the formation of aldosterone to the same extent. The conversion factor of these three steroids to aldosterone is in the order of 5 to 7% in 2 hours of incubation irrespective of the three dose levels tested. Thus, the presence of an hydroxyl group in 21, as in desoxycorticosterone, in 21 and 11 as in corticosterone, or their absence from 21 and 11 as in progesterone, was not reflected in the amount of aldosterone formed. This observation suggests that oxygenation in the 18 position might be the limiting factor in the biogenesis of aldosterone.

The present results find strong support in the work of Ayres who demonstrated a comparable conversion of C^{14}-progesterone, C^{14}-desoxycorticosterone and C^{14}-corticosterone to C^{14}-aldosterone upon incubation of these steroids with strippings of ox adrenal cortex (28), and concluded that they might represent a major, though not necessarily the only pathway of aldosterone biosynthesis. The data presented in this paper agree fully with this conclusion. As a whole, they also support the concept of biogenesis formulated by the Worcester Group (29) since they do not exclude the existence of a common precursor of mineralo and glucocorticoids, but only emphasize that the nature of the end product of its metabolic transformation is fully dependent on the existence of its 18-oxygenation solely in the glomerulosa or of its 17-hydroxylation solely in the fasciculata-reticularis.

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FUNCTIONAL ZONATION OF THE ADRENAL CORTEX: SITE OF ACTH ACTION

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ABSTRACT

When ACTH or corticotropin-peptides were added to the incubation media of beef adrenal slices composed chiefly of glomerulosa tissue they did not significantly affect the total production of corticosteroids nor that of aldosterone. The same corticotropin preparations markedly increased the production of total corticosteroids and of hydrocortisone in the incubation media of slices composed of fasciculata-reticularis cells.

In the presence of fasciculata-reticularis slices, ACTH promoted the conversion of cholesterol to hydrocortisone. In the presence of glomerulosa slices, ACTH neither promoted the conversion of cholesterol to aldosterone nor did it increase the conversion of 21-hydroxypregnenolone and progesterone to aldosterone.

NUMEROUS attempts have been made to localize the site of action of ACTH within the different cellular zones of the adrenal cortex. These investigations, which in majority were based on the observation of histological changes of the cortex following ACTH stimulation, have led some to believe that ACTH controls principally the growth and the function of the fasciculata-reticularis cells (1, 2). But relying on similar histological criteria, a number of investigators have emphasized that the zona glomerulosa is far from being independent of pituitary and ACTH influence (3–6).

By a different approach, Sonenberg (7) has obtained evidence that after the administration of I^{131} labelled ACTH preparations to rats there was a definite preference for the radioactivity to concentrate at the level of the two inner cellular zones of the cortex.

The relative ease with which the zona glomerulosa of beef adrenal can be separated from the fasciculata-reticularis (8) made it possible to reinvestigate this problem by a more direct approach. Consequently, it was decided to study the production of corticosteroids by the glomerulosa and the fasciculata-reticularis of beef adrenal incubated in the presence of ACTH.

METHODS

The methods used in the following study are essentially the same as those described in detail in the preceding paper (8). However, in order to obtain sufficient amounts of

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steroid material without increasing the time involved in the processing of a greater number of slices, the period of incubation was extended to four hours. In each experiment the different corticotropins were weighed and dissolved in Krebs-Ringer bicarbonate medium at a concentration of 2 I.U. per cc. After the usual half-hour of preincubation, the slices were transferred to a set of clean beakers. The required amounts of corticotropin in solution were poured onto the slices which were gently mixed to insure a uniform absorption of the hormone. The incubation media were then adjusted to the proper volumes (10 cc per gm. of tissue incubated) with Krebs-Ringer bicarbonate solution. In the experiments where both ACTH and corticosteroid precursors were used, these steroids were first redissolved in small amounts of the incubation media to which the slices were then transferred. The ACTH was added and the media adjusted to their proper volumes.

RESULTS

In a single experiment, glomerulosa, fasciculata and reticularis slices were incubated separately in order to study the responsiveness of the zona reticularis to ACTH stimulation. The quantitative estimations of Δ^4-3 ketosteroids, α-ketolic steroids, 17-hydroxycorticosteroids, (Porter Silber chromogens) and sodium-retaining activity in the crude lipid extracts of their respective incubation media are presented in Table 1.

TABLE 1. EFFECT OF ACTH ON THE PRODUCTION OF STEROID MATERIAL BY THE DIFFERENT CELLULAR ZONES OF BEEF ADRENAL CORTEX

	Glomerulosa				Fasciculata				Reticularis			
	μg./100 gm. of tissue/hr.				μg./100 gm. of tissue/hr.				μg./100 gm. of tissue/hr.			
	U.V.[1]	BT.[2]	P.S.[3]	M.A.[4]	U.V.	BT.	P.S.	M.A.	U.V.	BT.	P.S.	M.A.
Controls	1030	750	20	130	450	375	270	4	400	270	180	0
ACTH	1290	880	50	115	1800	1560	1070	1	1020	740	490	0
% Increase over Control	25	17	150		300	310	295		155	170	172	

[1] Maximal absorption at 2400 Å.
[2] Blue Tetrazolium reaction.
[3] Porter-Silber reaction.
[4] Mineralocorticoid activity in the Simpson-Tait's assay expressed in μg. of aldosterone.

These data already suggest that the main site of action of ACTH on corticoid production is to be found in both the fasciculata and the reticularis zones, whereas ACTH has a very weak or almost no action on the production of steroids by the isolated zona glomerulosa. Thus, in the presence of ACTH, the formation of Δ^4-3 ketosteroids, α-ketolic and 17-hydroxysteroids by the fasciculata and the reticularis was increased by about 300% and 160% above that of the respective controls.

In the glomerulosa, the production of Porter Silber reacting material only was definitely enhanced by ACTH (150% above control value). But one should consider that with or without added ACTH this material was recovered from the incubation media of glomerulosa slices to the extent of only 3–4% of the total amount found in the incubation media of both fasciculata and reticularis slices. It is therefore probable that the significant increase of Porter Silber reacting compounds induced by ACTH in the incubation media of the glomerulosa slices merely reflects the action of ACTH on the production of 17-hydroxysteroids by fasciculata cells incompletely separated from the glomerulosa slices.

Finally, ACTH did not affect the production of sodium-retaining material which, as previously shown, was found exclusively in the incubation media of glomerulosa slices.

CHROMATOGRAPHIC STUDIES

A series of studies was then undertaken to investigate the action of ACTH on the production of aldosterone and corticosterone by the zona glomerulosa and on the production of hydrocortisone and corticosterone by the fasciculata-reticularis. In these experiments, ACTH was added to the incubation media of both parts of the cortex at the dose level of 1.0 I.U. per gm. of tissue incubated for four hours. The results are presented in Table 2. They show that under the conditions of these experiments, the production of aldosterone by the zona glomerulosa was equal to 71 ±16.0 (S.D.) μg./100 gm. of tissue/hour, and to 84 ±19.5 after the addition of ACTH ($P > .1$). The production of aldosterone was not significantly affected by ACTH. Conversely, in the presence of ACTH, the amounts of

TABLE 2. ACTION OF ACTH[1] ON THE PRODUCTION OF ALDOSTERONE AND CORTICOSTERONE BY THE GLOMERULOSA AND ON THAT OF HYDROCORTISONE AND CORTICOSTERONE BY THE FASCICULATA-RETICULARIS OF BEEF ADRENAL

Steroid measured		Controls		ACTH[1]	
		μg. of steroid/100 gm. of tissue/hour			
Glomerulosa	Aldosterone	83 65 55 57 98 72	71 ±16.0[2]	101 64 63 105 98 72	84 ±19.5
	Corticosterone	96 109 109 21	84	153 112 142 62	117
Fasciculata-reticularis	Corticosterone	14 18 18	16	28 43 36	35
	Hydrocortisone	55 58 38 41 40 52	48 ± 8 6	106 189 114 117 187 245	160 ±55.0

[1] ACTH Nordic-Biochemicals: 1 I.U./gm. of tissue incubated 4 hours. ACTH increases significantly ($P < .001$) the production of hydrocortisone, but has no action on the production of aldosterone ($P > .1$).

[2] Standard Deviation.

hydrocortisone produced by the fasciculata-reticularis (160 ±55.0 μg./100 gm. of tissue/hour) were very significantly increased above the control values (48 ±8.6 μg.; $P < .001$).

In these experiments, the glomerulosa incubated in the absence of ACTH was observed to produce more corticosterone (mean of four values: 84 μg./gm. of tissue/hour) than the fasciculata-reticularis (mean of four values: 16 μg./gm. of tissue/hour). ACTH increased the production of this hormone by only 27% in the incubation media of the glomerulosa slices and by 112% in those of the fasciculata reticularis. The validity of these results regarding the lack of action of ACTH on the production of corticosterone by the glomerulosa is limited by the observation of great variations in the production of this hormone by both parts of the cortex upon extension of the incubation period (see discussion).

Farrell has reported that some corticotropin-peptides prepared by Bell increase the production of aldosterone in the adrenal vein blood of decapitated dogs (9).

Table 3 and 3a present the results of a series of incubations in which $\gamma_1+\alpha_2$, $\alpha_1+\alpha_2$ and δ_1, corticotropins were added to the incubation media of glomerulosa and fasciculata slices at dose levels varying between 0.01 and 12.8 I.U./gm. of tissue incubated/four hours. The total Δ^4-3 ketosteroids were measured in the crude lipid extracts of the incubation media in the three experiments; the total α-ketolic steroids only in two; aldosterone and hydrocortisone were estimated after paper chromatography. The re-

TABLE 3. ACTION OF CORTICOTROPIN-PEPTIDES[1] ON THE PRODUCTION OF CORTICOSTEROIDS BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL

Cortico-tropin (I.U./gm. of tissue)	Fasciculata-Reticularis (μg./100 gm. of tissue/hr.)			Glomerulosa (μg./100 gm. of tissue/hr.)		
	Crude lipid extract		Hydrocortisone	Crude lipid extract		Aldosterone[2]
	Δ^4-3 ketosteroids[2]	BT. Reacting compounds		Δ^4-3 ketosteroids[2]	BT. reacting compounds	
Control	320	400	40	1000	640	99
$\gamma_1+\gamma_2$ .2	600	630	108	1200	1100	103
.8	920	800	150	1200	1000	133
3.2	1200	1070	216	1330	1000	150
12.8	1560	1160	363	1600	1400	130
Control	373	431	47	720	530	83
δ_1 .2	1030	890	195	940	540	83
.8	1200	980	253	920	690	89
3.2	1510	1170	345	1140	1025	123
12.8	1860	1350	387	1200	985	130

[1] Corticotropin-Peptides Lederle. Fraction $\gamma_1+\gamma_2$: Lot No. 1079-1. Fraction δ_1: Lot No. 1079-24.

[2] Measured at 2400 Å.

TABLE 3a. ACTION OF CORTICOTROPIN-PEPTIDES[1] ON THE PRODUCTION OF CORTICOSTEROIDS BY THE GLOMERULOSA AND THE FASCICULATA-RETICULARIS OF BEEF ADRENAL

Corticotropin I.U./gm. of tissue	Fasciculata-Reticularis (μg./100 gm. of tissue/hr.)		Glomerulosa (μg./100 gm. of tissue/hr.)	
	Δ^4-3 ketosteroids[2]	Hydrocortisone	Δ^4-3 ketosteroids[2]	Aldosterone
0.01	210	90	440	90
0.1	450	190	520	100
1.0	890	250	670	100
10.0	1100	370	720	95
Control	140	60	380	91

[1] Corticotropin-Peptides Lederle: Fraction $\alpha_1 + \alpha_2$.
[2] Measured at 2400 Å.

sults show that only in the incubation media of fasciculata-reticularis did these three ACTH peptides markedly increase the production of Δ^4-3 ketosteroids and α-ketolic steroids, and that the degree of the response was proportional to the dose of corticotropins added. The production of hydrocortisone increased from control values of 40, 47 and 60, to 363, 387 and 370 μg./100 gm. of tissue/hour in the presence of the greatest dose of each corticotropin. As opposed to these results, the production of total Δ^4-3 ketosteroids and of α-ketolic steroids, as well as that of aldosterone by the glomerulosa slices was little affected by any one of the three ACTH peptides over the range of dose levels used, and in no case could a dose-response relation be established.

Since the work of Stone and Hechter on perfusion of beef adrenals, it has been generally recognized that one of the main actions of ACTH on corticosteroid biogenesis may lie in its control of an enzymic sequence essential for the conversion of cholesterol to Δ-5 pregnenolone and, consequently, for an increased formation of hydrocortisone and corticosterone (10). It was therefore considered of interest to study whether ACTH would promote the formation of aldosterone from cholesterol in the zona glomerulosa, or would promote the conversion of cholesterol to other corticosteroids in the fasciculata-reticularis exclusively. Hence, glomerulosa and fasciculata-reticularis slices were incubated in the presence of added cholesterol, 21-OH pregnenolone or progesterone, both with and without ACTH. The results of these experiments are reported in Table 4. They show that the production of aldosterone was unaffected by addition of cholesterol but was increased in the presence of 21-OH pregnenolone and progesterone. The addition of ACTH did not affect the conversion of 21-OH pregnenolone or progesterone to aldosterone, nor did it promote an increase of aldosterone in the presence of cholesterol. The production of hydrocortisone by the fasciculata-reticularis was increased above the control values by 170% in the presence of ACTH alone, and by 320 to 490%

TABLE 4. PRODUCTION OF ALDOSTERONE BY THE GLOMERULOSA AND OF HYDROCORTISONE BY THE FASCICULATA-RETICULARIS OF BEEF ADRENAL INCUBATED WITH PRECURSORS IN BOTH THE PRESENCE AND THE ABSENCE OF ACTH

		Controls	ACTH[1]	Cholesterol[2]		21-OH Pregnenolone[2]		Progesterone[2]	
				Alone	+ACTH	Alone	+ACTH	Alone	+ACTH
µg./100 gm. of tissue/hour.	Aldosterone	88 ± 11[3]	99 ± 16	115	113	310	240	330	320
				102	115	299		320	294
	Hydrocortisone	51 ± 7	136 ± 38	81	240	117	210	520	483
				62	340	144		600	742

[1] Nordic Biochemicals: 1 I.U./100 mg. of tissue incubated 4 hours.
[2] Added at the dose level of 10 µg./100 mg. of tissue incubated 4 hours.
[3] Standard deviation of the mean.

in the presence of both ACTH and cholesterol. Finally, the increased production of hydrocortisone promoted by *progesterone* was *not affected* by the addition of ACTH.

DISCUSSION

As early as 1940 Swann (11) expressed the view that hormones controlling mineral metabolism could be produced by the zona glomerulosa since the histological aspect of this zone as well as mineral metabolism are little affected by hypophysectomy. Conversely, the two inner zones of the cortex could be the site of production of hormones controlling carbohydrate metabolism since the profound disturbance of this metabolism after hypophysectomy is accompanied by marked atrophy of these zones. This view was fully supported by the data of Bergner and Deane (1) who showed that the histochemical changes observed in the adrenal cortex of ACTH-treated rats were mostly located within the fasciculata-reticularis. More recent works have demonstrated that prolonged treatment of the dog with glucocorticoids is followed by a marked decrease in the rate of production of hydrocortisone in the adrenal vein blood and accompanied by atrophic changes in the fasciculata-reticularis without detectable alteration of the zona glomerulosa (2). Since it is known that after cortisone administration the synthesis of pituitary ACTH is almost fully depressed (12) the over-all impression gained from this more direct approach is that in the dog also the site of action of ACTH is most likely limited to the cells of the fasciculata-reticularis.

The independence of the zona glomerulosa from pituitary control has been challenged by a number of histological studies. Thus Selye and Stone noted even in short term experiments in rats that a large dose of ACTH can stimulate growth of the zona glomerulosa (3). In hypophysectomized rats, Lever has observed a regression of all three cortical zones and conversely marked changes in the histological aspect of the zona glomerulosa after ACTH administration (4). Complete or almost complete depletion of sudanophilic elements of the zona glomerulosa was also observed by Wexler in rats treated with certain ACTH preparations (5). Following extensive histological studies in mice, Chester Jones expressed the view that the zona glomerulosa has probably no specific function in the elaboration and secretion of mineralocorticoids (6). From these data alone it appears difficult to draw definite conclusions on the site of action of ACTH on the adrenal cortex.

Comparing the production of aldosterone by the zona glomerulosa and that of hydrocortisone by the fasciculata-reticularis, the present work strongly suggests that the action of ACTH on corticosteroid biogenesis is mostly demonstrable in the presence of cortical slices made up entirely of fasciculata-reticularis cells. This conclusion is based on observations that three of the well-recognized effects of ACTH on corticoid production are clearly and exclusively demonstrable in the presence of fasciculata-reticularis slices: 1) in the presence of ACTH the production of hydrocortisone by the fasciculata-reticularis was markedly enhanced; 2) a linear response relationship between the dose of ACTH used and the increase of hydrocortisone production was demonstrable; 3) ACTH promoted the increase of hydrocortisone formation in the presence of added cholesterol.

The possible action of ACTH on the biogenesis and secretion of aldosterone has fostered a number of investigations. Singer has demonstrated that after hypophysectomy the secretion of aldosterone in the adrenal vein blood of rats is decreased to 25% of normal, and that the secretion of this hormone is returned to normal by treatment with ACTH (13). In hypophysectomized dogs, secretion of aldosterone in the adrenal vein blood effluent was observed by Farrell to be reduced by 1/3 of the normal value (15). The same author was the first to provide evidence that the rate of aldosterone secretion is depressed to about 30% in decerebrated dogs (16) and brought back to normal or above normal values after intravenous perfusion of crude beef diencephalon extracts (17). A less marked but comparable effect on hydrocortisone secretion was recorded. In the same animal preparation a linear response could be obtained between doses of δ_1 and β corticotropin-peptides and the amounts of aldosterone secreted in the adrenal vein blood (9). Perfusion of beef adrenals (18) or incubation of rat

(19) as well as human (20) adrenals with ACTH has led to some increase in the production of aldosterone although the results obtained with rat adrenals have often been found to vary with the preparation of ACTH used (19). Finally, the production of aldosterone by beef adrenal slices was increased by certain posterior pituitary extracts which had only a questionable action on the production of hydrocortisone; in these experiments ACTH had the opposite effect (21). These apparently conflicting results have led to the hypothesis, first expressed by Farrell (9), that a humoral factor different from ACTH might be responsible for the regulation of aldosterone secretion. The existence of such a factor, its presence in widely varying amounts as a contaminant of many pituitary hormones and (possibly) hypothalamic extracts, and the degree to which its action on aldosterone secretion might be interfered with by the action of ACTH on glucocorticoid secretion, could explain the apparent contradiction of results obtained in studies of the action of ACTH on aldosterone secretion.

The results of the present investigation show: 1) that none of the corticotropic preparations studied had significant action on the production of aldosterone by the isolated zona glomerulosa, 2) that the production of this hormone is unaffected by addition of cholesterol alone or by both cholesterol and ACTH, and finally, 3) that ACTH has no demonstrable action on the conversion of possible precursors of aldosterone such as pregnenolone and progesterone.

In these experiments corticotropin-peptides were not able to increase the production of aldosterone by more than 50% of the control values. Such an increase is felt to be too close to the possible range of variation in the production of aldosterone by the controls to be of significance. The lack of action of $\delta - 1$ corticotropin on the production of aldosterone may seem surprising since *in vivo* the same preparation was demonstrated by Farrell to increase markedly the secretion of this hormone in the adrenal vein effluent of decapitated dogs, and that in both his and the present work the quantities used lay in the same range. It is difficult to explain this result solely by a lack of sensitivity of the *in vitro* method, since previous work has demonstrated a two to threefold increase of aldosterone secretion by whole slices of beef adrenal cortex incubated in the presence of posterior pituitary preparations (21).

To postulate a loss of potency of δ_1 corticotropin which was kept in a solid state at 5° C for a few weeks would provide an explanation which we do not think likely. In a preliminary experiment, undertaken with the aim of assessing crudely the possible differences of potency of these 3 corticotropin-peptides on the production of aldosterone and hydrocortisone, an incubation was performed using each preparation at one single dose level (2 I.U./gm. of tissue). In this experiment, which is not reported quantitatively here, the production of aldosterone was increased roughly 100%

above the control values by the three corticotropins used. These results could not be reproduced when each of these corticotropins was tested in the experiment reported here, despite the dose range used (0.01–12.8 I.U./gm. of tissue).

The data presented strongly support the concept of functional zonation of the adrenal cortex, as first established by Deane, by offering direct proof of the main action of ACTH on the two inner zones of the adrenal cortex. They cannot be interpreted as supporting or disproving the existence of a humoral factor controlling the secretion of aldosterone by the zona glomerulosa, the existence of which, as a possible contaminant of pituitary preparations, is suggested by *in vivo* studies (9).

The final proof that the secretion of corticosteroids by the zona glomerulosa is totally independent of ACTH might rest with studies on the production of corticosterone by this zone of the cortex. Preliminary data suggest that ACTH might have an action both on the production of corticosterone and on the conversion of cholesterol to corticosterone throughout the adrenal cortex though, possibly, to a greater extent in the fasciculata-reticularis.

However, in the present study it has often been difficult to measure corticosterone after incubation of the slices for four hours with or without added ACTH. It was repeatedly noticed that at the end of 2 hours of incubation the amounts of corticosterone recovered from the incubation media were approximately equal in both parts of the cortex. However, by increasing the time of incubation to 4 hours or more, corticosterone progressively disappeared from the media. This was observed whether in the absence of added precursors or in the presence of progesterone. In one incubation with this steroid, the amounts of corticosterone produced by the glomerulosa and the fasciculata-reticularis were respectively equal to 280 and 1560 μg./100 gm. of tissue after 4 hours of incubation, whereas no corticosterone was recovered from the incubation media of glomerulosa, and only 60 μg./100 gm. of tissue from that of the fasciculata-reticularis after 8 hours of incubation.

Perfusions of beef adrenal have shown that ACTH increases the production of corticosterone (22). On the other hand, the present data indicate both the importance of corticosterone as a possible precursor of aldosterone in the zona glomerulosa (8), and the insignificant action of ACTH on aldosterone biogenesis. The existing data admit at least two different possibilities: either that both aldosterone and the fraction of corticosterone produced by the glomerulosa belong to a pathway of steroid biogenesis which by-passes cholesterol and is slightly or not at all affected by ACTH; or that throughout the cortex, corticosterone is under ACTH control—which could imply that this steroid is not an obligatory pathway in the biogenesis of aldosterone.

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METABOLISM OF THYROXINE DURING PROLONGED KIDNEY CORTEX INCUBATION[1]

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ABSTRACT

A study has been carried out of the metabolic changes in thyroxine, as the hormone maintained the oxygen consumption of rat kidney cortex slices incubated for three days at 5°C. Tetraiodothyroacetic acid (Tetrac) and iodide were rapidly produced, with triiodothyroacetic acid (Triac) and small amounts of a thyroxine conjugate appearing later. When Tetrac was added instead of thyroxine, conjugation was more rapid, but no Triac was formed. Addition of Triiodothyronine (Trit) resulted in Triac formation, as well as maximal production of both glucuronic and sulfuric acid conjugates. No Trit was encountered, even with careful bidimensional chromatography; thus, renal deamination and decarboxylation of Trit must dispose of it as fast as it is produced from thyroxine. No correlation could be obtained between production of any metabolite of thyroxine and its effect on kidney cortex metabolic rate. These results suggest that there is a response of some labile factor in the cell rather than production of an "active" thyroxine derivative.

IT HAS previously been shown (1) that, during prolonged incubation of rat kidney cortex at 5° C, added thyroxine had the striking effect of maintaining oxygen consumption of the tissue for as long as 72 hours. This action was especially marked in the presence of alanine or proline, and was completely eliminated by the addition of tryptophan. The present study is concerned with the metabolism of the thyroxine. Since there is considerable current interest in the possible production of "active" forms of the hormone, 3,5,3'-triiodothyronine (Trit) and 3,5,3',5'-tetraiodothyroacetic acid (Tetrac) were also studied.

METHODS

The experimental conditions were the same as those reported by Lindsay and Barker (2). Slices of kidney cortex from thyroidectomized rats were placed in Warburg vessels in Ringer-alanine-glucose or Ringer-proline-glucose and held at 5° C, oxygen consumption being measured at 37° C before incubation (Day 0) and after 1, 2 and 3 days. To the 10 μg. thyroxine present per ml. of medium was added radioiodine-tagged thyroxine (Abbott), about 100,000–250,000 cpm per flask. Comparative determinations showed no effect of this much radioactivity on the oxygen consumption. Variations in this gen-

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eral procedure included terminating the incubation at earlier times, incubation for various lengths of time before addition of thyroxine, or substitution of a thyroxine-free solution. In addition, radioiodine-tagged Trit or Tetrac together with carrier in amounts equimolar to 10 μg. thyroxine/ml. were substituted for the thyroxine.[2]

Immediately following the desired incubation, the contents of each set of duplicate vessels were pooled and centrifuged at 0° C. The clear supernatant medium was poured off and kept cold for counting and chromatography. The tissue pellet was washed three times with cold Ringer solution, being separated each time by centrifugation at 0° C. Each sample was then repeatedly extracted with small portions of methanol-1% ammonium hydroxide in a small Potter-Elvehjem glass homogenizer kept in ice. The pooled extracts were filtered and used either directly, or after concentration at 40° C under reduced pressure. Although the latter procedure has been found not to alter the distribution of iodinated compounds, we have preferred to start with sufficient radioactivity to avoid this extra step of handling. It was necessary to use the incubation medium without concentration to avoid adsorption of organic iodine on the large quantity of solid. *We have already reported that electrolytic desalting results in deiodination of thyroxine* and related compounds (3).

Before chromatography, aliquots of the tissue extracts and incubation media were evaporated and counted dry with a thin end-window Geiger-Müller tube. Suitable quantities were then chosen for treatment by ascending chromatography with the following solvent mixtures: butanol-water-acetic acid (78:17:5), butanol-water-ammonium hydroxide (250:178:72), tertiary amyl alcohol-water-ammonium hydroxide (50:40:10), or collidine-water (100:35) in an atmosphere of ammonia from concentrated ammonium hydroxide. Quantitative Geiger-Müller scanning of the activity in the developed but unstained chromatogram was carried out along a marked grid. Autoradiograms were obtained on Kodak No-Screen x-ray film. Chemical demonstration of spots was carried out with sprays of ninhydrin for amino acids or diazotized sulfanilic acid for phenols (4). Iodine-containing compounds were revealed with a ceric sulfate-arsenious acid stain (5). After qualitative identification, each substance present was expressed quantitatively in terms of percentage of the total radioactivity added as thyroxine, Tetrac, or Trit.

RESULTS

Metabolism of thyroxine. The three-day maintenance of kidney cortex metabolic rate in the presence of thyroxine is demonstrated in Figure 1A for the composite of several experiments involving incubation in Ringer-alanine-glucose. The control vessels (B.) showed good respiration for the first day, but fell off rapidly after that time. There was a striking contrast when thyroxine was present in the incubation medium throughout the entire three-day period.

The distribution of radioactivity in the tissues and incubation solutions of these experiments is shown in Figure 2 for various lengths of time. The hormone was rapidly taken up by the tissue, 32% being thus located after only 20 minutes at 37°, and 65% after 1 hour. Metabolic alterations of the thyroxine were also rapidly occurring, with a small amount of Tetrac appearing in both tissue and solution after 20 minutes, as well as 5% of inorganic iodide. These actions continued during the rest of the first hour.

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[2] Generous quantities of these substances were provided by Dr. R. Pitt-Rivers of the National Institute for Medical Research, Drs. R. I. Meltzer and R. L. Kroc of the Warner-Lambert Research Institute, Dr. A. E. Heming of the Smith Kline and French Laboratories, and Dr. H. L. Fevold of the Baxter Laboratories.

By the end of one day of incubation (2 hours at 37° C and 23 hours at 5°), the total tissue radioactivity had begun to fall. The distribution of activity was essentially unchanged, but the amount now present as acetic acid derivatives ("Ac") had increased, especially in the tissue. At this time, Triac first clearly appeared in the tissue. As the incubation was extended through four days, the amount of these acetic acid derivatives increased, as well as the proportion present as Triac. Tissue activity was evidently responsible for the necessary deamination and decarboxylation, since there was always more acetate derivative in the tissue than in the solution. Careful bidimensional chromatography consistently failed to reveal the presence of Trit at any time, from 20 minutes to three days (Fig. 3). Since there was always more Tetrac than Triac, we originally concluded that the former was the source of the latter, as well as of the activity found as iodide. However, it will be shown later that added Tetrac yielded no Triac, whereas added Trit did.

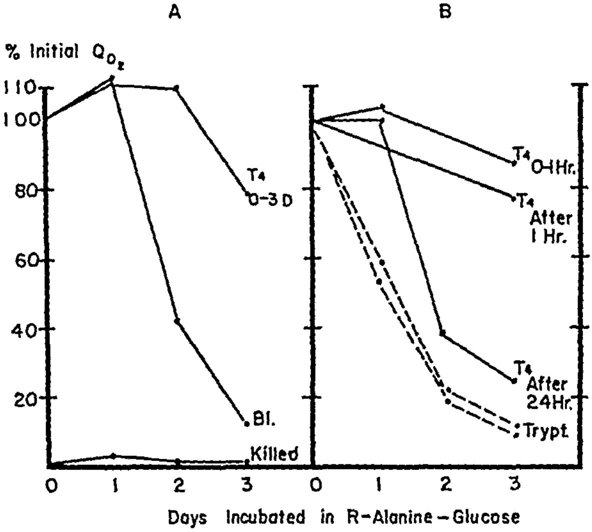

FIG. 1. Oxygen consumption of rat kidney cortex slices in Ringer-alanine-glucose solution. Measurements made at 37° C on days 0, 1, 2, 3; incubation otherwise at 5° C. "T_4" indicates solution containing 10 µg. L-thyroxine/ml.; "Bl" control solution. "Trypt" solutions contained added tryptophan, thyroxine flasks the lower dotted line. See text for further explanations.

In some experiments, it was possible to see two thin lines of radioactivity on the autoradiograms between the origin and the iodide. As the scanning was ordinarily done on acid-developed chromatograms of incubation solutions, these compounds were included in the thyroxine fraction. More exact counting indicated that these amounted to 4% of the total activity at most.

By analogy with other results to be presented, (see Fig. 8) it is believed that these are conjugates of thyroxine.

Experimental changes in the exposure of kidney slices to thyroxine were introduced, as shown in Figure 1B. It can be seen that, when thyroxine was present in the solution only for the first hour of incubation (at 37° C) or when the hormone was not added until after that hour, the 3-day maintenance of metabolism was nearly as great as when thyroxine was present throughout the entire time. In contrast, withholding thyroxine *until after* the first 24 hours of incubation gave results no different from the control solution. When tryptophan was added, the thyroxine action was completely blocked. The tissue subjected to 100° C for 10 minutes did not consume significant amounts of oxygen at any time.

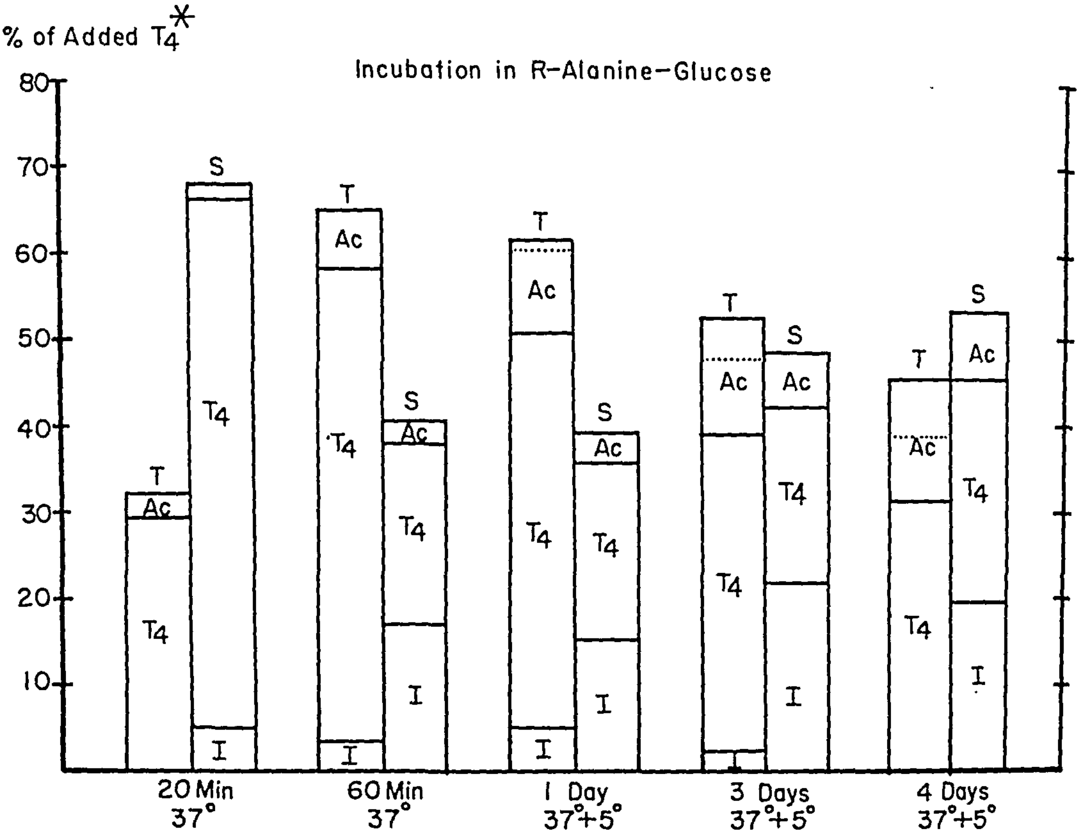

FIG. 2. Metabolism of added radiothyroxine under certain of the conditions shown in Fig. 1 The first two columns show the redistribution of radioactivity between solution (S) and tissue (T) after 20 and 60 minutes at 37° C, the remainder at various times of longer incubation at 5° C combined with respiration measurements at 37° C. Fractions are indicated as follows:

T_4, thyroxine; Ac, Tetrac plus Triac (latter above dotted line when present); I, inorganic iodide.

Figure 4 shows that tryptophan did not alter the metabolism of thyroxine. Even delaying the addition of thyroxine until after one day produced the quantitative differences one would expect from the shorter time available

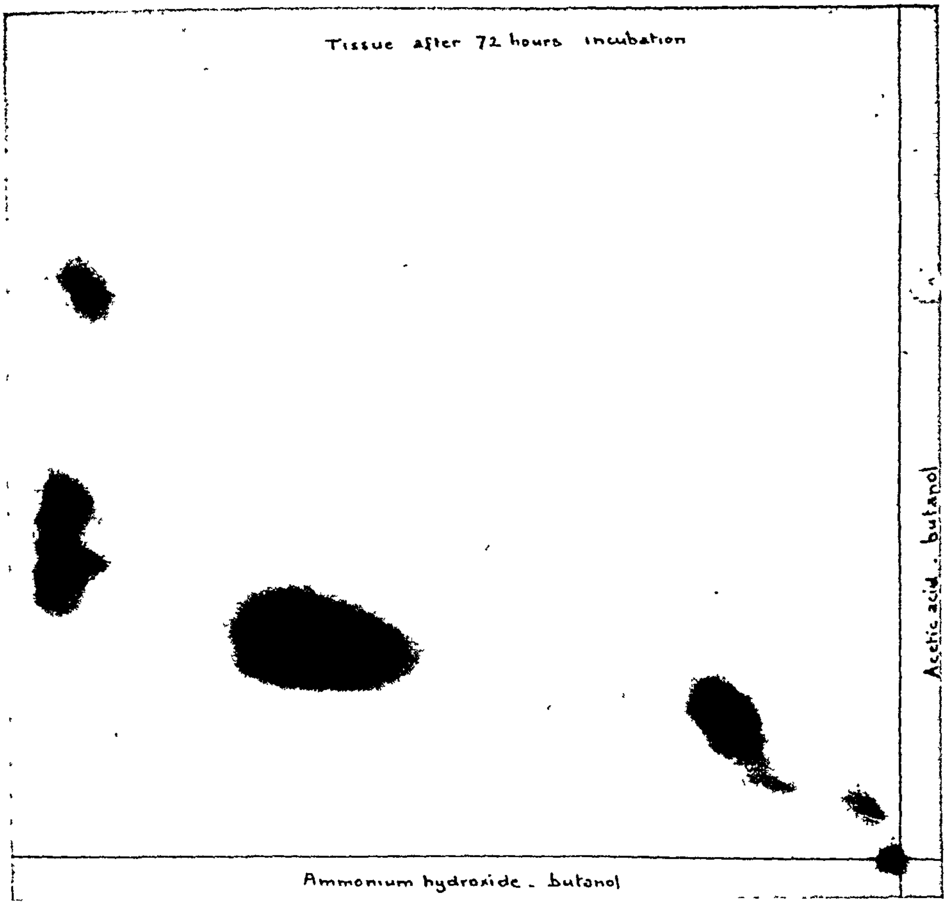

FIG. 3. Autoradiogram of bidimensional chromatogram of extract of kidney tissue slices incubated with radiothyroxine for 72 hours. Origin lower right, solvents as shown. References identified spots present as:

Triac
Tetrac Thyroxine
Iodide
Origin

for tissue action on the hormone, namely, a greater proportion of thyroxine in the tissue, and lower levels of Tetrac and iodide. When thyroxine was present only for the first hour, the tissue retained 60% of the radioactivity, mostly as thyroxine itself (Fig. 5). During the succeeding 3 days, there was a great loss of this thyroxine into the new solution added without thyroxine. This lower tissue hormone level resulted in a smaller production of Tetrac. In all these instances, it is apparent that metabolic changes were produced in the thyroxine as an amino acid and that the production of Tetrac or Triac cannot be considered an "activation," nor does the presence of Trit seem to be necessary.

The results with the heat-denatured tissue were surprising (Fig. 4).

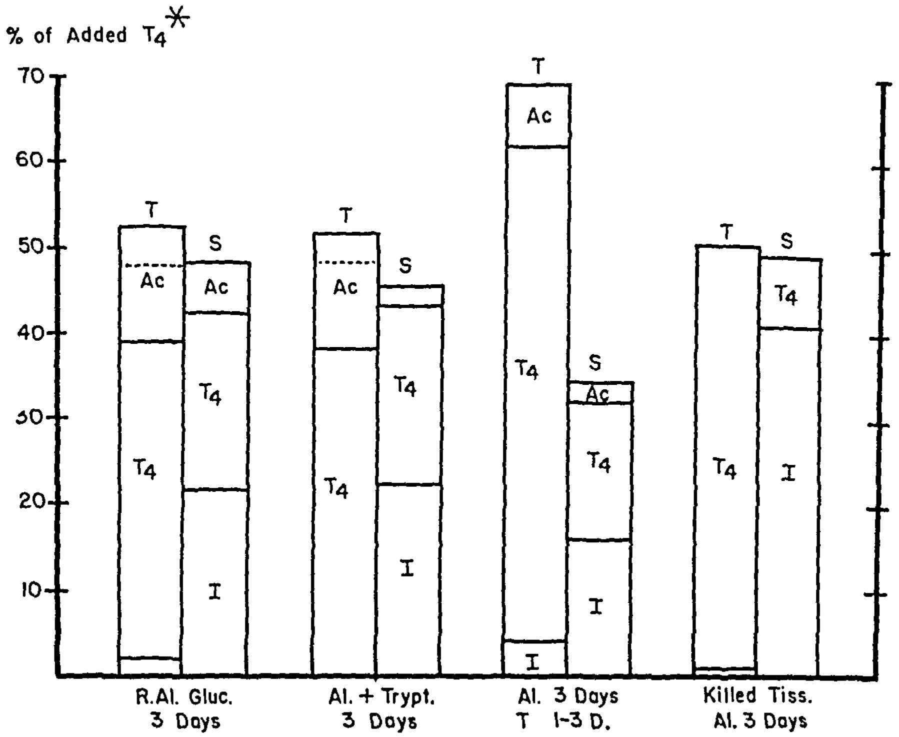

FIG. 4. Metabolism of radiothyroxine during kidney cortex incubation under certain of the conditions shown in Fig. 1. Symbols as in Fig. 2.

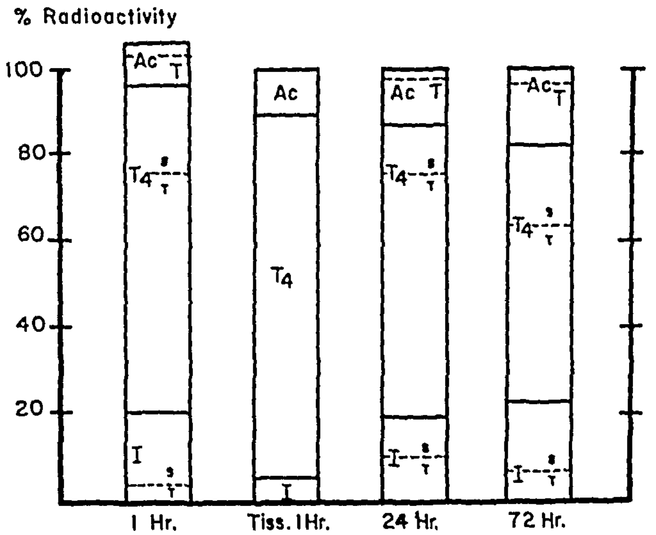

FIG. 5. Redistribution of the radioactivity present in kidney slices after 1 hour's exposure to radiothyroxine at 37° C during a subsequent 3 days' incubation in Ringer-alanine-glucose. Column 1: original distribution of activity in tissue (T) and solution (S) at the end of 1 hour at 37° C. Column 2: tissue portions of column 1 expanded to 100%. Columns 3 and 4: redistribution and metabolism of radioactive materials originally present in the tissue (column 2) after incubation at 5° C for 24 and 72 hours, respectively. Symbols as before: T_4, thyroxine; Ac, Tetrac plus Triac; I, inorganic iodide.

There was a rapid "uptake" of thyroxine, and even after 3 days, the washed killed tissue contained more than four times the outside concentration of thyroxine. Furthermore, twice as much iodide had been formed as with the viable tissue. Extended study of this phenomenon, to be reported elsewhere, has shown that thyronine is one principal product of deiodination.

When proline was the added amino acid, the metabolism of thyroxine was very nearly the same as in the presence of alanine. Some of these results are presented in Figure 6, for comparison with alanine. There was approximately the same amount of thyroxine present in the tissue at the end of three days in both, 43% in proline and 35% for alanine. Tryptophan blocked thyroxine maintenance, as before, but did not interfere with the production of Tetrac, Triac and iodide. Again, delaying the addition of thyroxine until after one day of incubation, completely eliminated its effect on kidney metabolism, but introduced no more than the quantitative decrease in thyroxine products expected from the shorter time.

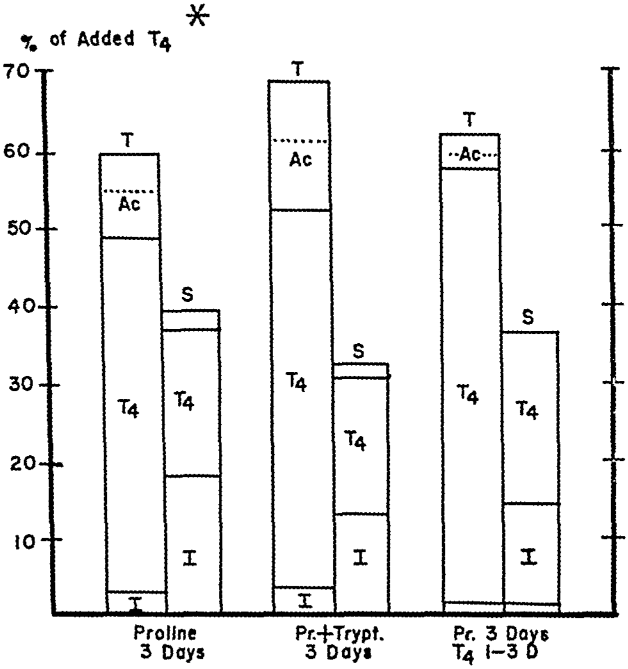

FIG. 6. Metabolism of added radiothyroxine (T_4*) by kidney cortex slices incubated in Ringer-proline-glucose (Columns 1, 3) or Ringer-proline-glucose with tryptophan (Column 2). In column 3, thyroxine was present only after the first 24 hours of incubation. Symbols designating partitioned radioactivity as in Fig. 2.

Metabolism of Tetrac. Since Tetrac appeared as an early metabolic product of thyroxine, its effect was next studied. Figure 7A shows that Tetrac maintained oxygen consumption of incubated kidney cortex slices in much the same manner as thyroxine: 119% of the initial Q_{O_2} value after 24 hours, 101% after 48, and 78% after 72.

Chromatography of the tissue and medium after 72 hours of incubation (Fig. 7B) revealed most of the radioactivity in the former to be the original Tetrac, with a very small amount of iodide and another spot of low R_f in alkaline solvents. In addition to a smaller amount of Tetrac and a larger quantity of iodide, the medium contained enough of the low R_f compound to account for 10% of the total radioactivity (see Fig. 8). When the incubation solution was chromatographed using collidine-ammonium hydroxide, an R_f of 0.31 was obtained, strongly suggesting a glucuronic acid conjugate. A preparative chromatogram was therefore run, using butanol-ammonium hydroxide and the spot was eluted with methanol-ammonium hydroxide. After condensation *in vacuo* below 40° C, the material was taken up in acetate buffer at pH 5 and incubated 2 hours at 37° C with a liver β-glucuronidase (Warner-Chilcott Ketodase®). Under these conditions there was a small amount of deiodination, but chromatography in collidine-ammonia and butanol-ammonia showed disappearance of the glucuro-conjugate and appearance of Tetrac. It can thus be concluded that 12% of the Tetrac originally present was conjugated with glucuronic acid, most of this reappearing in the solution. The second conjugate which can be seen faintly in Figure 8 is believed to be a sulfate, although the amounts present were too small to permit isolation.

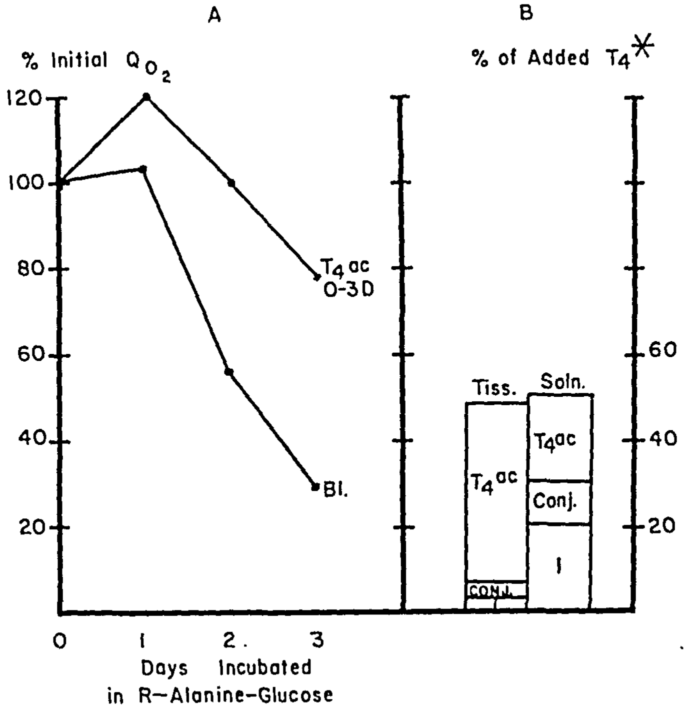

FIG. 7. Tetrac addition to kidney slices. A. Maintenance of oxygen consumption with added Tetrac (T_4 ac). B. Metabolism of radioactive Tetrac during 3 days' incubation. "Conj." indicates glucuronic acid conjugate of Tetrac.

Repeated *mono-* and bidimensional chromatograms were run with in-

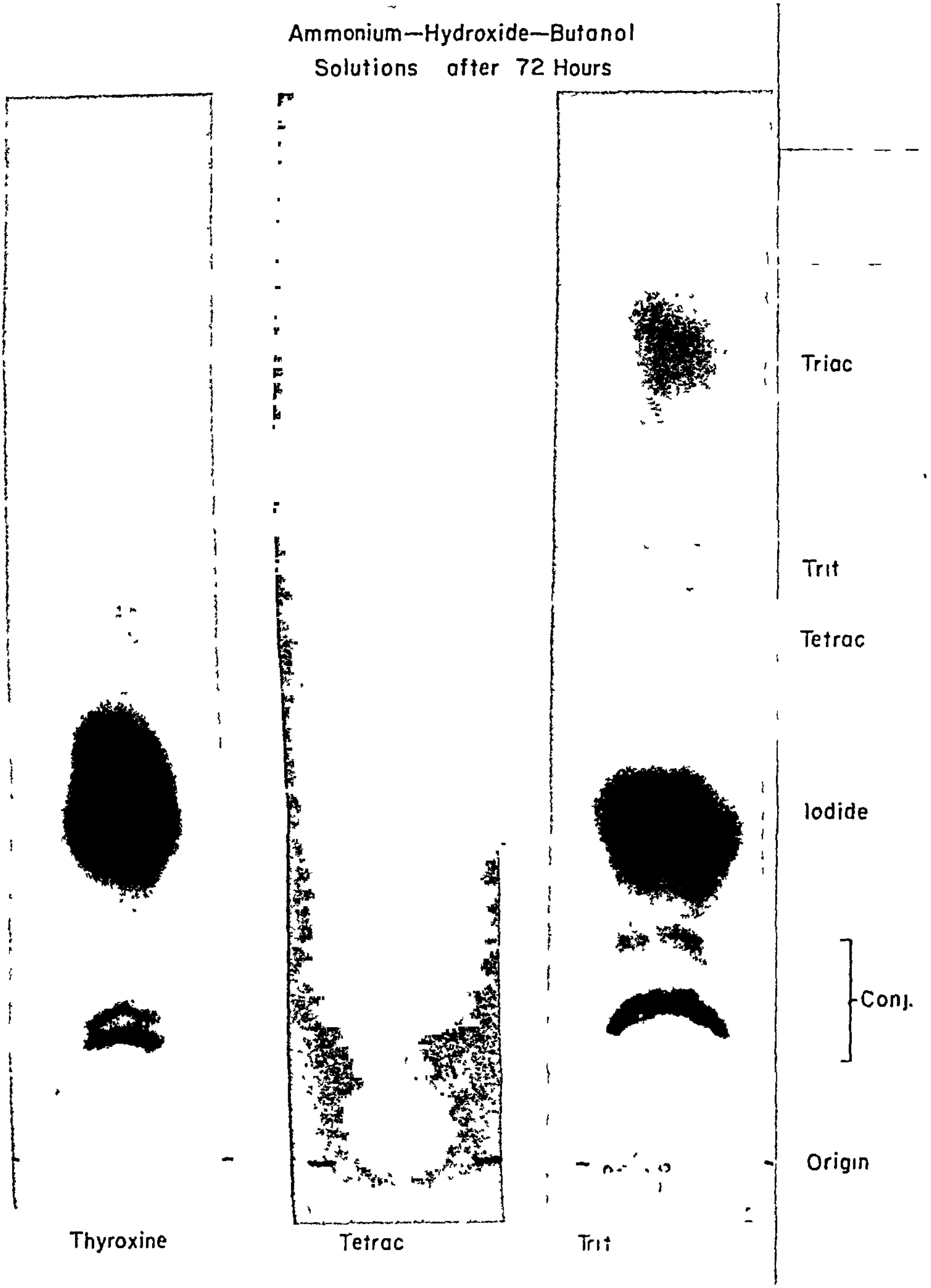

FIG. 8. Autoradiograms of three different solutions after 72 hours' incubation with rat kidney cortex slices, chromatographed in butanol-ammonium hydroxide. Exposure times were not consistent from one strip to another, but relative amounts of radioactivity in the conjugates and inorganic iodide are comparable. The spots are indicated upwards from the origin at the bottom: conjugates (Conj.), inorganic iodide, Tetrac in the thyroxine and Tetrac media, Trit and Triac with Trit medium.

cubation solution and tissue extracts. In no case was it possible to see a Triac spot even on bidimensional autoradiograms, interpreted as meaning that less than 2% of the Tetrac could have been partially deiodinated to Triac. The appreciable quantities of Triac formed in the thyroxine experiments, therefore, could not have come from Tetrac.

Just as with thyroxine, tryptophan blocked the Tetrac maintenance entirely, but did not alter either deiodination or glucuro-conjugation. Thus, this is additional evidence that the oxygen consumption effect of thyroactive materials is exerted independent of the metabolism of these.

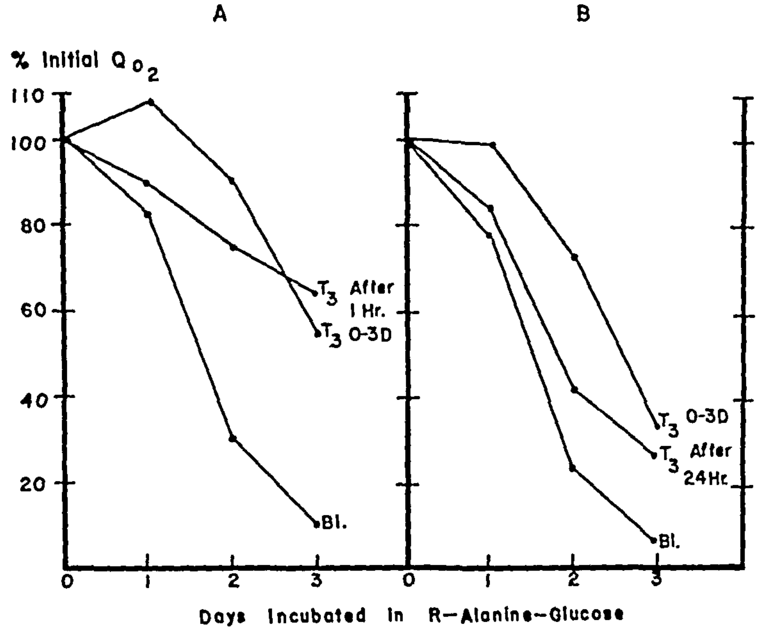

FIG. 9. Effect of Trit (T_3) on maintenance of oxygen consumption of kidney slices in Ringer-alanine-glucose. Other symbols as in Fig. 1.

Metabolism in the presence of 3,5,3'-Triiodothyronine (Trit). Although Trit was not actually encountered at any time during incubation of the kidney cortex slices with thyroxine, the formation of Triac from thyroxine but not from Tetrac made it imperative to evaluate Trit. Figure 9 shows that this product also was capable of producing oxygen consumption effects when it was added at the start of incubation or after the preliminary hour at 37° C. Maintenance, although clearly better than the blank, was not as effective as with thyroxine.

Trit is known to be more active than thyroxine *in vivo*, that is, the same metabolic stimulation can be obtained with one-quarter to one-tenth as much of the former. This does not occur *in vitro*, since the response to various concentrations of Trit is the same as with thyroxine. Trit has also been shown to be metabolized and eliminated from the body more rapidly than thyroxine (6). Figure 10 presents studies on the metabolism of Trit by kidney slices showing 60% penetration by 60 minutes, no more rapid than that of thyroxine. This value had fallen to 50% by 72 hours. Triac was formed at about the same rate as Tetrac plus Triac in the case of thyroxine for the first 48 hours, although the 72-hour figure was greater with Trit. Iodide also appeared from both in approximately equal amounts. There was only 37% of free Trit remaining after 72 hours, in contrast to 57% of the thyroxine. The disparity between the amounts left in the tissues was not as great, being 30% for Trit and 36% for thyroxine. It is not certain that any of these differences is adequate to explain the poorer *in vitro* maintenance with Trit, or the greater metabolic effects obtained *in vivo*.

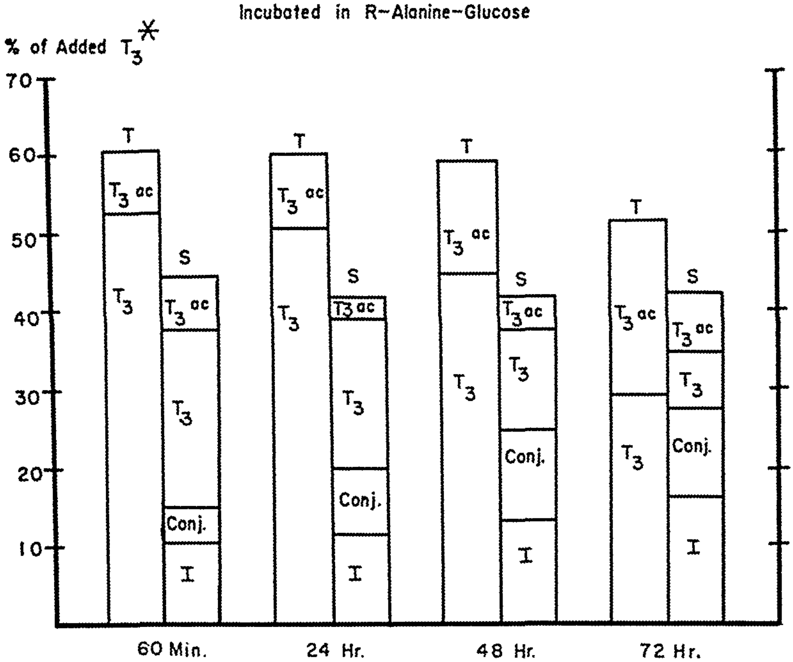

FIG. 10. Metabolism of radioactive Trit (T_3^*) after various times of incubation as in Fig. 8. Other symbols: T_3, Trit; T_3 ac, Triac; Conj., glucuronic and sulfuric acid conjugates of Trit; I, inorganic iodide.

Chromatograms of the incubation solutions in butanol-ammonium hydroxide revealed two spots between the origin and iodide (Fig. 8). Run in collidine-ammonium hydroxide, treatment with bacterial β-glucuronidase (Sigma) eliminated the spot of R_f 0.3 and increased the amount of Trit. This material was, therefore, the glucuroconjugate of Trit. The second material, closer to iodide, appeared to be the sulfoconjugate of Trit, similar to that found in rat bile after administration of Trit to the animal (7). About 5% of the added Trit was conjugated by these two mechanisms during the first hour, increasing to 22% by the end of the experiment. Neither conjugate was found in the tissue itself, nor was iodide.

Autoradiograms of the 72-hour bidimensional chromatograms presented an unexpected picture. From the tissue extracts, besides the anticipated Trit and Triac, there was obtained a faint spot of R_f 0.84 in butanol-acetic acid and 0.84 in butanol-ammonium hydroxide. An acid metabolite of

Trit other than Triac of 3,3′-diiodothyroacetic is suspected. In the incubation solution, besides Triac, the conjugates mentioned, and iodide, there was a very faint spot of R_f 0.81 in butanol-acetic and 0.20 in butanol-ammonium hydroxide, for which no identification can be offered.

DISCUSSION

There was an increase of the different metabolites of thyroxine during the time incubation of rat kidney slices. Similar results were obtained with Trit. The acetic acid derivatives first accumulated in the tissue, then the excess passed into the incubation medium. Although Yamazaki and Slingerland (8) did not encounter any acetic acid derivatives, these have also been identified as resulting from homogenate action on thyroxine by Tomita *et al.* (9). Other components, such as the conjugates and iodide, passed rapidly out of the tissue and were found in much higher concentration in the solution.

It has been well established that the liver can conjugate thyroxine and related compounds (10). Taurog *et al.* (11) demonstrated that kidney was also capable of a smaller level of glucuro-conjugation of thyroxine. The present studies not only confirm this renal activity but show that Tetrac is more readily conjugated than thyroxine, and Trit the most extensively of the three. The autoradiograms strongly suggest that the last compound readily forms a sulfoconjugate as well as the glucuronic. The only obvious structural basis for these differences in extent of conjugation would be in the case of Trit, where removal of the 5′-iodine would give greater freedom of approach to the 4′-hydroxyl group.

Thyroxine maintenance of respiration in these experiments could be achieved if the hormone was added after the first hour at 37° C, but not after 24 hours at 5°. The metabolites produced from thyroxine were the same under all these circumstances. This strongly suggests that thyroxine had an important effect on something in the kidney cells which remained intact for about one hour at 37° C but was lost by 24 hours at the lower temperature, rather than involving some "active form" of the hormone (12). This concept is supported by two additional pieces of evidence. Added tryptophan completely blocked any thyroxine maintenance, but did not interfere with the production of Tetrac, Triac and iodide. Furthermore, addition of Tetrac instead of thyroxine did not give significantly different results. Other experiments, not reported here, have ruled out Triac as peculiarly active.

The source of the Triac encountered during incubation with thyroxine requires some consideration. Tetrac always appeared first, and always was present in greater amounts than Triac, leading one to suspect Tetrac as the immediate source. However, added Tetrac yielded no Triac, whereas Trit did, and in considerable quantitity, equal to Tetrac plus Triac from thyroxine. Although no trace of Trit was found during the thyroxine experiments, it seems probable that it must have been formed and immediately deaminated and decarboxylated. There always remains the unprovable possibility that Tetrac as produced from thyroxine is more labile than exogenous Triac added to the incubation medium.

From many of the figures, it is evident that deiodination is a prominent feature of the metabolism of thyroxine, as well as of Tetrac and Trit, by kidney tissue. The most unusual characteristic was that heat-killed slices showed twice as much deiodination at the end of 3 days as did viable tissue. This point will be pursued more in detail elsewhere, but it can be noted that non-enzymic deiodination has been shown for liver and pancreas by Lissitzky *et al.* (13). Larson *et al.* (14) and Tata (15) reported thermolabile deiodination by kidney, and by brain and muscle, respectively. Under our conditions, the summated deiodination processes, if more than one, were less active in viable tissue.

Considerably more iodide appeared in every experiment than could be accounted for on the basis of triiodo-compounds formed. Presumably, complete deiodination of the respective parent compounds must have occurred, since the sensitive ceric-arsenious stain revealed no iodine spots corresponding to non-radioactive 3,5-iodinated compounds which might have been produced. Although thyronine has been identified as the principal product from heat-killed deiodination of thyroxine, no identification of Pauly-reactive compounds has been possible in the experiments on living tissue, because of the small quantities available, and the relative insensitivity of the color reaction as compared to the iodide test.

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THE EFFECT OF TOLBUTAMIDE ON THE DIABETOGENIC ACTION OF SOMATOTROPIN[1]

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ABSTRACT

The administration of tolbutamide to dogs by mouth prevents the diabetogenic response which ordinarily occurs with the daily intramuscular injection of somatotropin.

THE mechanism whereby the daily administration of somatotropin to the dog results in the development of a temporary diabetic state (idiohypophyseal diabetes) which is followed by a permanent diabetic state (metahypophyseal diabetes) is still unknown (1, 2, 3). All available evidence supports the hypothesis that somatotropin induces an increased peripheral need for insulin with a resultant increase in the production of insulin by the beta cells of the pancreas and that when the rate of insulin utilization exceeds the rate at which it can be manufactured, the beta cells undergo degranulation and hydropic degeneration. Thus, the idiohypophyseal diabetes is attributed to a relative insufficiency in the capacity of the pancreas to meet the increased peripheral requirements for insulin while the metahypophyseal diabetes which persists after the cessation of the somatotropin injections is attributed to the destruction of the beta cells (1, 2, 3).

The diabetogenic action of extracts of the anterior pituitary gland is inhibited in fasted, fat-fed, insulin-treated and phlorhizin-treated animals (4, 5, 6). These measures presumably reduce the peripheral requirements for endogenous insulin and thereby prevent the excessive stimulation otherwise produced by the administration of somatotropin. Since the hypoglycemic action of tolbutamide in most species is believed to be due, in part at least, to the secretion of insulin by the beta cells in the pancreas (7, 8, 9), it became of interest to evaluate the effect of this compound on the diabetogenic action of somatotropin.

METHODS AND RESULTS

Male dogs, weighing from 10 to 12 to 17 kilograms, were given a daily intramuscular injection of from 3 to 10 mgm. somatotropin per kilogram body weight for variable

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periods of time before, during and after the administration of tolbutamide. The tolbutamide was given daily by mouth in a single dose which varied from 50 to 100 mg. per kilogram body weight. All dogs were given a constant daily diet and any food that was not eaten by 4:30 p.m. was removed and weighed. Venous blood samples were taken between 8 and 9 every morning before the animals were fed or given any of the compounds under study. The daily urine excretion was collected quantitatively. The concentrations of glucose in the blood and the quantity excreted in the urine was determined by Nelson's method (10).

Illustrative of the effect of tolbutamide are the data depicted in Figure 1. As noted by others (1, 2, 3), within a few days after the start of the daily injections of somatotropin, the dog developed a marked hyperglycemia and glycosuria which persisted throughout the period during which somatotropin was given. By the third day after the daily injections of somatotropin were stopped, the blood sugar was back to the pre-treatment concentration and the glycosuria ceased. Subsequently, however, when the animal was given 50 mg. tolbutamide daily by mouth, the administration of the previously diabetogenic dosage of somatotropin produced only a small transient increase in the blood sugar concentration and a transient glycosuria.

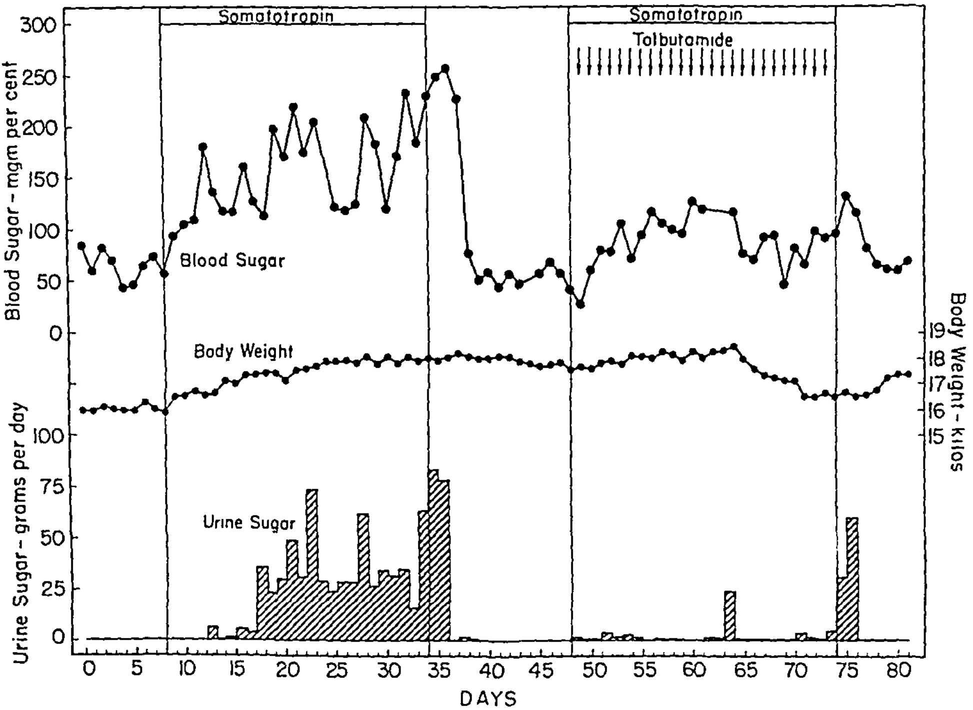

FIG. 1. Effect of tolbutamide on diabetogenic action of somatotropin. 5 mg. somatotropin per kilo injected intramuscularly daily during periods noted. During the second period of somatotropin administration, 50 mg. tolbutamide per kilo given daily by mouth.

The significance of these and similar data obtained with 8 other dogs is difficult to evaluate in view of the evidence that both somatotropin and tolbutamide produce beta cell stimulation. Yet tolbutamide produces a hypoglycemic response and somatotropin a diabetogenic response when given alone. The prevention of the diabetogenic response when both agents are given at the same time suggests that excessive stimulation of the beta cells of the pancreas may not be the principle determinant in the diabetogenic response to somatotropin.

Acknowledgments

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THE ACUTE EFFECT OF SALTS ON THE URINARY EXCRETION OF RADIOIODIDE IN MICE[1]

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ABSTRACT

The acute effect of various ions on the urinary excretion of radioiodine was investigated by measuring the radioiodine excretion in the urine of mice injected with I^{131} subcutaneously and given 0.517 milliequivalent of various salts through the gastric route.

Sodium, potassium, rubidium, chloride, bromide, iodide ions increased the excretion of radioiodine, while lithium, calcium, ammonium, carbonate and acetate ions did not. Glucose and water had no effect.

Since only those salts releasing one or more highly dissociated ions did increase the excretion of radioiodine, this effect appears to be due to an increased concentration of ions in the body.

ADMINISTRATION of sodium chloride to mice increases the urinary excretion of the iodide ion to an extent such that a relative iodine deficiency and a stimulation of the thyroid gland may occur (1). The chemical property in sodium chloride responsible for this effect was investigated in mice by administering sodium, chloride and other ions and measuring the amount of radioiodine excreted in the urine.

MATERIALS AND METHOD

The mice used were C_3H or albino of either sex, aged 2–9 months. The tables indicate the strain, sex and age used for each experiment. Prior to the experiment, they had been fed for 3 weeks on a low iodine diet composed of 89% corn meal, 10% dried brewer's yeast and 1% calcium carbonate and containing only traces of sodium and chloride (2), to minimize the urinary excretion of iodide in controls. Then the animals were given 0.2 ml. of an aqueous solution containing 0.517 milliequivalent of whatever salt was under investigation (30 mg. in the case of sodium chloride). The controls received an equal volume of water. The solution—or water—was introduced into the stomach either by tubing, using a polyethylene tube "intramedic" No. 10, the external diameter of which was 0.6 mm., or by injection through the muscular wall of the stomach. Immediately afterwards, the mice received a subcutaneous injection of 10 μc. carrier free I^{131} in 0.2 ml. water. Each animal was then placed in a 250 ml. beaker fitted with a wire mesh floor allowing the urine to settle at the bottom and was given access to distilled water

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but not to food. Two and a half hours later, the mouse was anaesthetized with ether in the beaker. Bladder and thyroid gland were removed. The thyroid was homogenized in 10 ml. 2N NaOH. The beaker containing the urine and the bladder were washed with 5 ml. 2N NaOH and 10 ml. water. In both cases 1 ml. of the solution was poured on a watch glass-supported lintine filter disc, dried at room temperature and counted with a Geiger-Müller counter. Standards were made for thyroid and urine by adding a known amount of radioiodine to sodium hydroxide solutions. The counts were corrected for background and expressed as a per cent of the injected dose of radioiodine.

In one experiment the renal clearance of radioiodine from the blood was measured. Two groups of mice were treated as indicated above, except that one group was sacrificed after 1 hour and the other after 3 hours. In addition, the radioiodine content of blood (obtained by heart puncture) at sacrifice time, was measured. The clearance over the 2-hour interval between the 1st and 3rd hour after the beginning of the experiment was calculated according to the formula

$$U_3 - U_1 \cdot \frac{\mathrm{Ln}B_3 - \mathrm{Ln}B_1}{B_3 - B_1} \cdot \frac{1}{120} \text{ ml. cleared per minute,}$$

where U_1 and U_3 are the amount of I^{131} excreted in urine during the first hour and the first 3 hours respectively, and B_1 and B_3 the I^{131} concentrations in blood at 1 and 3 hours respectively.

RESULTS

Lithium chloride increased the urinary excretion of radioiodine (Table 1). The effect of this salt may be attributed to the chloride ion since three other lithium salts (carbonate, acetate and fluoride) did not significantly influence this excretion.

The two sodium salts tested, namely sodium phosphate and sodium bicarbonate increased the urinary excretion of radioiodine (Table 1). The effect of sodium bicarbonate can be attributed to the sodium ion since neither the carbonate ion of lithium carbonate nor the hydrogen ion of acetic acid had any effect. Thus both chloride and sodium ions were efficient in increasing iodide excretion. However, when used separately, neither one nor the other of these ions increased radioiodine excretion to the same extent as when used jointly in sodium chloride.

Several salts containing neither sodium nor chloride ions were also capable of influencing iodide excretion (Table 1). Thus, rubidium carbonate increased it, presumably through the rubidium ion. Potassium bicarbonate increased iodide excretion ten times, but the effect could not be proved statistically due to the small numbers of survivors. Ammonium carbonate had no effect, calcium acetate and acetic acid decreased radioiodine excretion. All halogenides tested except fluoride increased radioiodine excretion. Iodide was significantly more potent than the other halogenides. Glucose had no influence on this response.

Administration of large amounts of water had no effect. Table 1 shows the results obtained in animals given food and water up to the time of injection, but other groups denied access to water or both food and water during the night preceding the experiment did not show any effect of water

TABLE 1. THE ACUTE EFFECT OF 0.517 MILLIEQUIVALENT OF VARIOUS SALTS ON THE URINARY EXCRETION AND THYROID UPTAKE OF RADIOIODINE IN MICE

Treatment	Number of mice	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
(C_3H male 2 months-old mice)			
Control	8	3.7 ± 1.2	23.1 ±2.8
Lithium carbonate (Li_2CO_3)	7	5.5 ± 1.6	*7.9 ±4.5*
(Albino male 4 months-old mice)			
Control	8	9.9 ± 2.3	12.7 ±1.6
Lithium acetate	7	13.7 ± 5.5	9.1 ±1.0
(C_3H male 2.5 months-old mice)			
Control	6	4.7 ± 1.1	26.8 ±3.1
Sodium bicarbonate ($NaHCO_3$)	7	*37.2 ± 4.6*	23.4 ±2.9
Di-sodium phosphate (Na_2HPO_4)	7	*33.6 ± 4.9*	19.7 ±1.2
Sodium chloride	7	*53.8 ± 3.6*	*17.1 ±1.4*
(C_7H male 5 months-old mice)			
Control	8	3.2 ± 0.9	23.4 ±1.7
Potassium bicarbonate ($KHCO_3$)	3	30.9 ±15.2	*10.8 ±2.6*
Sodium chloride	8	*56.2 ± 2.8*	*13.5 ±1.2*
(Albino male 3 months-old mice)			
Control	8	12.7 ± 3.1	11.8 ±0.7
Rubidium carbonate (Rb_2CO_3)	8	*38.9 ± 2.6*	11.0 ±2.0
Sodium chloride	8	*47.8 ± 3.8*	10.4 ±1.2
(Albino male 4 months-old mice)			
Control	8	9.9 ± 2.3	12.7 ±1.6
Ammonium carbonate $(NH_4)_2CO_3$	3	16.0 ± 5.0	13.1 ±4.2
Sodium chloride	7	*50.2 ± 5.3*	14.4 ±2.3
(C_3H male 5 months-old mice)			
Control	8	3.2 ± 0.9	23.4 ±1.7
Calcium acetate	7	*1.1 ± 0.3*	*9.7 ±1.5*
Acetic acid	8	*0.4 ± 0.1*	*7.5 ±1.5*
(C_3H female 9 months-old mice)			
Control	8	3.1 ± 0.6	10.4 ±3.6
Lithium fluoride	7	6.9 ± 2.4	11.4 ±5.3
Lithium chloride	7	*14.1 ± 2.5*	12.3 ±4.4
Lithium bromide	8	*17.3 ± 2.4*	11.0 ±4.6
Lithium iodide	8	*38.5 ± 5.0*	*0.14 ±0.04*
Sodium chloride	8	*55.6 ± 2.8*	7.1 ±3.2
Glucose*	8	3.4 ± 1.0	8.5 ±2.3
(C_3H male 3.5 months-old mice)			
Water 0.1 ml.	8	3.1 ± 0.7	15.3 ±2.7
Water 1.5 ml.	8	2.7 ± 1.0	15.7 ±1.3

The italicized values are significantly different from the respective controls.
* 0.517 millimoles.

either. (After injection of water, the animals were not allowed to drink water.)

The thyroid uptake of radioiodine was unchanged after administration of most of the salts, and was occasionally slightly diminished. But in mice given iodide, the thyroid uptake was drastically reduced and was only 1/79th of the uptake in controls.

A number of salts were found to be toxic. The toxicity of potassium bicarbonate and ammonium carbonate is reflected by the small number of animals surviving after 2.5 hours (Table 1). Hydrochloric acid, ammonium chloride, ammonium fluoride, sodium fluoride, potassium fluoride, were also tested, but were too toxic for conclusions to be reached regarding iodine excretion.

To investigate whether the increased excretion of radioiodine after administration of sodium chloride is due to a change in the functional activity of the kidney, the radioiodine clearance from blood was measured between the first and third hours following treatment. Table 2 shows that in sodium chloride treated mice the clearance was raised to 22 times the value in controls.

DISCUSSION

The respective roles of sodium and choride ions in raising the urinary excretion of iodide are clearly pointed out by the data obtained: both sodium and chloride ions increased the radioiodide excretion in urine (Table 1) and, therefore, both are responsible for the effect observed after administration of sodium chloride.

TABLE 2. EFFECT OF SODIUM CHLORIDE ON BLOOD RADIOIODIDE CLEARANCE BY THE KIDNEY

Treatment (Albino male 3 months-old mice)	Sacrifice time (hours)	Number of mice	% of injected I^{131} in		Blood I^{131} kidney clearance (ml./minute)
			Urine	1 ml. blood	
			Mean ±S.E.	Mean ±S.E.	
Controls	1	7	7.9 ±2.1	3.8 ±0.4	0.007
	3	7	11.2 ±2.8	3.7 ±0.5	
Sodium chloride, 30 mg.	1	7	17.4 ±4.3	3.0 ±0.5	0.152
	3	7	50.8 ±5.9	1.0 ±0.4	

The data obtained indicate that a similar effect could also be produced by ions other than sodium and chloride. However, the fact that some salts were not effective raised the question whether any particular chemical property is responsible for the effect. When the salts investigated were divided into those that do and those that do not increase iodine excretion, it appeared that the effective salts were those which introduced a large number of free ions in the body. As is well known, a well defined fraction of any salt in solution dissociates into ions. Thus, a large proportion[2] of sodium, potassium, rubidium, chlorine, bromine and iodine ions dissociate from the other moiety in the salts used. All of them increased the excretion of radioiodine. On the contrary, the salts formed from a poorly dissociated base[2] (e.g. ammonia) and a poorly dissociated acid[2] (e.g., hydrofluoric, carbonic, acetic), and which, therefore, liberate few ions, were inactive. Bases well dissociated like lithium and calcium hydroxides,[2] but which form poorly soluble salts with physiological anions (phosphate, carbonate)[3] were also inactive. The importance of the ionic concentration is also pointed out by the fact that sodium chloride which releases in solution 2 well dissociated ions was more potent than sodium bicarbonate or lithium chloride, which introduce only one free ion, respectively sodium or chloride. In addition, glucose whose ionization is negligible did not influence iodine excretion. Thus, it may be concluded that the urinary excretion of iodine is increased whenever the concentration of ion in the body increases. This view also accounts for the observation that physiological saline has little effect on the excretion of radioiodine (3).

Administration of iodide-I^{127} (as lithium iodide), in addition to raising the ionic concentration of plasma like other salts, has two effects which do not occur under treatment with the other salts. Firstly, the iodide ion which is administered with lithium iodide behaves exactly like the tracer which is being measured, and therefore "dilute" it. Secondly, in the presence of a pharmacological amount of iodine such as that administered (66 mg.), the metabolism of iodide (and therefore of radioiodide) may be modified not only quantitatively but also qualitatively.[4] The respective role of these three factors in increasing radioiodide excretion is not known. It is possible that the increase in plasma ionic concentration raises the excretion of radioiodide to the same extent as in lithium chloride or bromide treated mice, and that a further raise is produced by the "dilution" factor, by saturation of the iodide reabsorption capacity in the kidney tubule.

The mechanism by which sodium chloride increases the urinary excretion of radioiodide cannot be explained merely on the basis of changes in the concentration of plasma radioiodide resulting from alterations in the extrarenal metabolism of iodine (e.g. change in blood circulation or in inter/intracellular distribution) but involves a change in the functional activity of the kidney, since the clearance of the isotope was sharply increased (Table 2). This is in agreement with earlier observations (1) and with the increase in radioiodine clearance after administration of a number of sodium salts (chloride, bromide, iodide, bicarbonate) as well as of choline iodide reported by Halmi *et al.* (4).

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[2] Degree of ionization of normal solutions of NaOH: 0.73, KOH: 0.77, HCl: 0.784, NH_4OH: 0.004, HF: 0.070, H_2CO_3: 0.0017, CH_3COOH: 0.004, LiOH: 0.63, $Ca(OH)_2$ (N/16): 0.90 (5).

[3] Solubilities in cold water of Li_3PO_4: 0.039 gm./100 ml., $Ca_3(PO_4)_2$: 0.002 gm./100 ml., and $CaCO_3$: 0.0015 gm./100 ml. (5).

[4] Such a change has been observed in thyroxine metabolism (6): normally excreted in feces only, the hormone appeared in the urine after injection of large doses.

Effect of salts on radioiodine uptake by the thyroid

The occasional diminution in thyroid uptake observed after administration of salts may be attributed to various factors. In the case of compounds which do not increase the urinary excretion of radioiodine, such as lithium carbonate, calcium acetate and acetic acid, the diminution in thyroid uptake is probably due to the toxicity of the salts. This factor probably also accounts for the diminution of radioiodine excretion in calcium acetate and acetic acid treated mice. In animals given sodium chloride the occasional decrease in thyroid uptake may be due to urinary losses depriving the thyroid of radioiodine although toxicity may also play a role. The decrease in thyroid uptake reflects the decrease in blood radioiodine level. Both decreases are slight early after treatment as in these experiments, but are much more marked later on (1). The very low uptake observed in mice treated with lithium iodide comes from the I^{127} load which dilutes I^{131} and saturates the capacity of the thyroid to incorporate iodine into organic compounds.

In conclusion, the fact that only highly dissociated salts increased the urinary excretion of radioiodine, suggests that iodide excretion is influenced by the ionic concentration of plasma.

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DISTRIBUTION OF C^{14}-ESTRONE IN RATS AND DOGS[1] BEFORE AND AFTER PLASMAPHERESIS

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ABSTRACT

The present study was undertaken to demonstrate possible effects of plasmapheresis on the distribution and clearance of C^{14}-estrone in rats and dogs. Plasmapheresis was accomplished by removing $\frac{1}{3}$ of the animal's blood volume, substituting dextran or polyvinyl pyrrolidone solution for the plasma, and reinjecting the mixture. This procedure was repeated 5–6 times. The plasma protein concentration fell to less than 10% of the starting levels. In dogs following plasmapheresis the clearance of unconjugated radioactivity from the plasma did not differ materially from that in the same dogs before plasmapheresis. On the other hand, the glucosiduronate levels in the plasma were much higher following plasmapheresis than in the control experiments. Approximately twice as much radioactivity, primarily as glucosiduronates, appeared in the urine following plasmapheresis as before. In rats, distribution of radioactivity was determined in organs, excreta and blood 20 minutes following the injection of C^{14}-estrone. In control rats 84% of the radioactivity appeared in the gastrointestinal tract and liver (54% and 30%, respectively) compared to 42% (15% and 27%, respectively) in the rats following plasmapheresis in which the plasma was replaced with 6% dextran in saline. Interestingly, 25% of the radioactivity was found in the lungs of the rats after plasmapheresis as compared to 2% in the controls. The administration of polyvinyl pyrrolidone to rats following plasmapheresis resulted in the appearance of 41% of the radioactivity in the gastrointestinal tract and liver and 6% in the lungs. More radioactivity was present in some of the organs and fat of rats who had undergone plasmapheresis and given polyvinyl pyrrolidone than in the control rats or in the rats given dextran.

THE binding of estrogens to plasma proteins has been amply demonstrated (1), but the significance of this binding in the transport, distribution, or metabolism of these steroid hormones has not been ascertained. The study reported here represents an attempt to answer some of these problems and is based on the distribution of injected C^{14}-estrone in normal rats and dogs before and after plasmapheresis.

MATERIALS AND METHODS

Male and female Sprague-Dawley rats (250–425 gm. in weight) were anesthetized with sodium pentobarbital (25 mg. per kg.) given intraperitoneally. The carotid artery

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and jugular vein were cannulated with small polyethylene catheters. Seven to ten ml. of blood were removed with a syringe from the arterial cannula, placed into a graduated centrifuge tube containing 0.1 mg. heparin, and centrifuged for 10 minutes. The plasma was removed and replaced by an equivalent volume of 6% dextran in 0.15M saline (Abbott) or 3.5% polyvinyl pyrrolidone (PVP-Macrose).[2] The mixture was reinjected into the jugular vein through the venous catheter. In the rats undergoing plasmapheresis this procedure was repeated five times. Plasmapheresis was accomplished in 1–2 hours. At the end of the plasmapheresis procedure 16-C^{14}-estrone (4,000,000 counts per minute /mg.) in 1 ml. of 6% dextran or PVP-Macrose was injected through the venous cannula and the cannula washed through with a small amount of plasma substitute. Each rat was injected with 125,000 counts per minute. The rats were sacrificed by exsanguination through the arterial catheter twenty minutes following the injection of the steroid. The organs and tissue specimens were then removed, blotted free of blood, and stored at −10° C until homogenized. The frozen tissues were weighed and homogenized in water either by hand or mechanically, and extracted three times with large volumes of absolute ethanol. The latter was evaporated by air to a small volume, part of which was plated and the radioactivity counted. Digestion of the protein residue with sodium hydroxide (0.1N) followed by extraction with chloroform failed to reveal any residual radioactivity. Control animals were operated on similarily, blood withdrawn, centrifuged and then reinjected. To some of these bloods concentrated solutions of dextran were added to produce a final concentration in the plasma of 2–6%, to others 90 mg. or less of dextran were added.

Plasmapheresis was performed in dogs anesthesized with 25 mg./kg. of sodium pentobarbital I.V. by removing 300–500 ml. of blood from the femoral artery, centrifuging the blood, and substituting the plasma with 6% dextran in saline. This procedure was repeated 5 to 6 times. The cells were reintroduced by intravenous infusion through the jugular vein. The dogs were then injected intravenously with 0.5–1.0 μc. of C^{14}-estrone (1 μc. per 370 g.).

The methods for extraction and hydrolysis of the plasma, urine, and erythrocytes with β-glucuronidase, at pH 1, and hot acid, and the determination of radioactivity in the urine and blood have been previously described (2). Total plasma proteins were determined by the method of Gornall *et al.* (3). The electrophoretic pattern was determined by free zone electrophoresis using a Perkin-Elmer Model 38 electrophoresis apparatus. All samples were dialyzed to equilibrium and the determination performed in barbiturate buffer (pH 8.6, ionic strength 0.1) according to standard techniques.

RESULTS

Since the major aim of the present study was to obtain as low a level of plasma protein as was possible, the most critical part of the experiment concerned itself with the rate of the plasmapheresis and the selection of a substitute for the removed plasma. Previous experiments had shown that the production of plasmapheresis in dogs over a prolonged period of time (one week) did not result in sufficient lowering of the plasma protein level. Hence, plasmapheresis was performed over a period of one to two hours, after which time the animals were injected with the radioactive estrogen.

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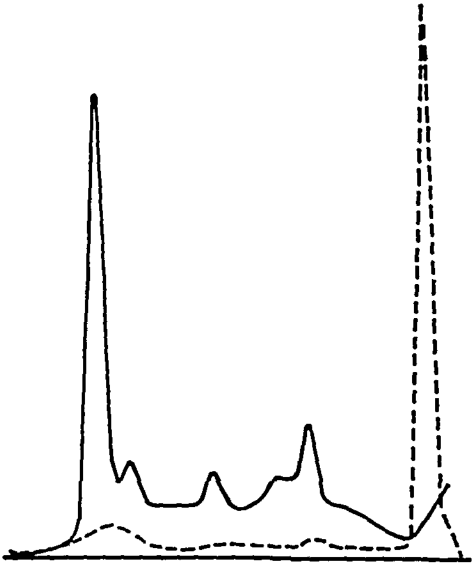
[2] PVP-Macrose was kindly supplied by Dr. P. Puetzer of Shenlabs Pharmaceuticals, Inc. It consisted of a solution of 3.5% PVP containing 0.65% NaCl, 0.042% KCl, 0.025% $CaCl_2$, 0.0005% $MgCl_2$, and 0.024% $NaHCO_3$.

In all experiments the plasma protein concentration fell to less than 10% (0.3–0.6 gm.%) of the original level (Fig. 1).

Initial studies showed that the replacement of plasma with physiological saline resulted in early death of the animals. Hence, in the experiments in dogs the plasma was replaced by 6% dextran in saline. The condition of the animals was good throughout the time of the experiments. Figure 2 shows the clearance of the mean unconjugated and conjugated radioactivity from the plasma of five dogs before and after the plasmapheresis, following the injection of C^{14}-estrone. The dogs served as their own controls. A period of at least three weeks elapsed following injection of radioactive estrogen before the plasmapheresis experiments were performed. The conjugated radioactivity was maintained at higher levels following plasmapheresis than in the control experiments. The conjugated radioactivity was primarily in the form of glucosiduronates.[3] This finding assumes further significance when examined in the light of the urinary data shown in Figure 3. The amount of radioactivity excreted in the urine during the six hours following injection of C^{14}-estrone was much higher following the plasmapheresis experiments than during the control period, in spite of the fact that the amount of urine produced following plasmapheresis was smaller than during the control period. As in the plasma, the major portion of the hydrolyzable and extractable radioactivity in the urine was in the form of glucosiduronates. Moreover, following plasmapheresis the amounts of the

FIG. 1. Electrophoresis pattern of plasma of Dog No. 2 before (solid line) and following (broken line) plasmapheresis. High peak on the right following plasmapheresis represents the injected dextran. Note great reduction in the amount of protein following plasmapheresis.



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[3] The term "glucosiduronates" has been used in this paper to refer to steroid metabolites conjugated with glucuronic acid. This nomenclature is in keeping with that published for "Rules of Carbohydrate Nomenclature," Chem. Eng. News, 31: 1776, 1953.

TABLE 1. MEAN AND RANGES (IN PARENTHESES) OF PERCENTAGES OF INJECTED DOSE OF C^{14}-ESTRONE IN ORGANS OF RATS BEFORE AND AFTER PLASMAPHERESIS

	Group A	Group B	Group C	Group D
Organs	Control rats No plasmapheresis (3M +2F)	Plasmapheresis plus dextran substituted for plasma (4M +2F)	Dextran added to plasma. No plasmapheresis (2M +2F)	Plasmaphersis plus PVP substituted for plasma (6M)
G-I tract plus liver	84% (80-89)	42% (18-64)	66% (54-80)	41% (31-48)
G-I tract	54% (43-71)	15% (8-26)	33% (22-43)	18% (6-25)
Liver	30% (15-38)	27% (10-38)	33% (15-52)	23% (15-37) (35%)*
Lung	2.0% (1-2.5)	25% (6-46)	7.0% (4.5-20)	6.0% (2-15)
Kidneys	1.4% (1.1-1.6)	2.3% (1.9-4.6)	2.8% (2.2-5.0)	3.5% (3.0-4.8)
Heart	0.3% (0.2-0.5)	1.4% (1.1-2.6)	0.8% (0.6-1.6)	1.8% (1.4-2.9)
Testes	1.4% (1.1-2.6)	0.8% (0.4-1.8)	1.6% (1.0-2.0)	1.7% (1.3-3.1)
Spleen	0.2% (0.1-0.3)	0.4% (0.3-0.7)	0.4% (0.3-0.5)	0.3% (0.1-0.5)
Adrenals	0.1% (0.09-0.12)	0.2% (0.1-0.4)	0.2% (0.1-0.4)	0.1% (0.09-0.12)
Bladder and urine	0.5% (0.3-0.7)	0.2% (0.1-0.4)	0.7% (0.4-1.1)	0.3% (0.2-0.6)
Uterus and adnexae	0.4% (0.2-0.6)	0.9% (0.7-1.1)	0.7% (0.3-1.4)	—
Muscle (c/m/g.)	253	163	245	231
Fat (c/m/g.)	476	515	348	796

* Percentage of injected radioactivity in the livers of two additional rats not shown in the Table.
M = male.
F = Female.
c/m/g = counts per minute per gram tissue.

urinary conjugated C^{14}-estrone metabolites were much higher than those present in the control urines.

Plasmapheresis in the rats presented several problems, primarily with regard to the choice of the plasma substitute. Examination of Table 1 reveals several interesting points. In the control animals (Group A) 84% of the injected radioactivity was present in the liver and the gastrointestinal tract 20 minutes after the injection of C^{14}-estrone. Following plasmapheresis in rats in which 6% dextran in saline was substituted for the

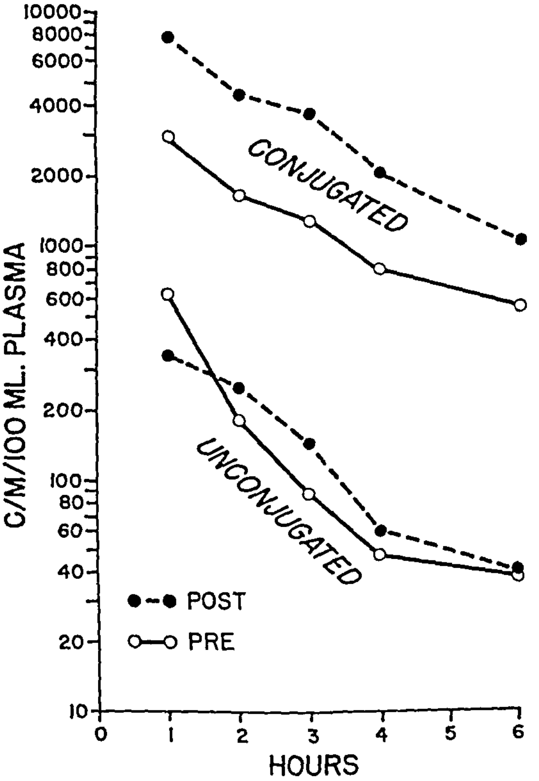

FIG. 2. Clearance of mean unconjugated and conjugated radioactivity (following the i.v. injection of C^{14}-estrone) from the plasma of dogs before and after plasmapheresis. Note much higher levels of radioactivity in conjugated (glucosiduronate) fraction following plasmapheresis.

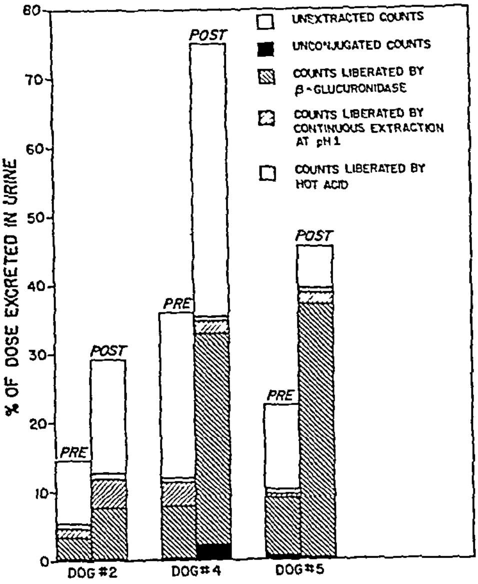

FIG. 3. Excretion of radioactivity, (following the i.v. injection of C^{14}-estrone) in the urine of 3 dogs before and after plasmapheresis.

plasma (Group B), the total amount of radioactivity present in the liver and the gastrointestinal tract was on the average only 42% (27% in the liver and 15% in the gastrointestinal tract). An extraordinary amount of radioactivity was present in the lungs (25%), with values as high as 46%. In the control rats only 2% of the radioactivity was present in the lungs.

It was important to ascertain whether the above findings were due to the lack of plasma proteins or a special effect of dextran (4, 5). Others have reported that in animals receiving dextran in saline as a substitute for the plasma the mortality rate was high, with most of the animals dying of respiratory of circulatory failure. Some respiratory embarrassment was present in our experiments in some of the rats that survived and in which dextran was used as a plasma substitute following plasmapheresis. Such rats in our experiments received from 600 to 1000 mg. of dextran. To test the effect of dextran without plasmaphoresis, a solution of dextran was concentrated three fold in a freeze-drying apparatus. This concentrated solution (0.5–3.0 ml.) containing 90–540 mg. was added to the centrifuged blood of normal rats before it was injected. It should be pointed out that the plasma was not removed from these bloods and that the cells were resuspended by gentle shaking of the sample before injection. The averaged results, Table 1 (Group C), show that 7% of the radioactivity was found in the lung. In one rat, who had received 540 mg. of dextran, 20% of the radioactivity was present in the lung, whereas in another identically treated rat over 4.5% was present in the lungs.

In order to obviate the untoward effect of dextran, plasmapheresis was performed on rats and the plasma replaced with PVP-Macrose. The latter is considered to be less toxic in the rat than dextran (4, 5). The results are shown in Table 1 (Group D). The rats seemed to tolerate PVP-Macrose better than dextran. The average radioactivity found in the gastrointestinal tract and the liver of these animals was 41% with approximately 30% in the liver. About 6% of the radioactivity was found in the lung, with the range being 1–15%. More radioactivity was found in the heart, kidneys and fat of the rats receiving PVP as a plasma substitute than in any other group.

The distribution of the radioactivity in the plasma and erythrocytes following the injection of C^{14}-estrone in rats showed some deviation from the controls when plasma substitutes were added (Table 2). More of the radioactivity was present in the unconjugated fraction and associated with the red blood cells in animals receiving plasma substitutes than in the control rats. The total amount of radioactivity present in the total blood volume of the largest rat at the time of exsanguination represented less than 10% of the injected radioactivity.

TABLE 2. MEAN RADIOACTIVITY IN PLASMA AND ERYTHROCYTES OF RATS 20 MINUTES FOLLOWING THE INJECTION OF C^{14}-ESTRONE (c/m/10 ml.)†

Group*	Unconjugated fraction	Glucosiduronate fraction	"Sulfate" fraction	Acid hydrolyzable fraction	RBC
A	460	300	120	100	680
B	1100	150	100	20	1100
C	900	300	110	50	1300
D	1100	80	100	110	1100

* Groups A, B, C and D are identical with those in Table 1.

† The radioactivity was measured in samples of blood the volume of which varied from 7–10 ml.

No binding of C^{14}-estrone could be demonstrated to 6% dextran in saline or PVP-Macrose under equilibrium dialysis techniques previously described (1).

DISCUSSION

From Figure 2 it is evident that plasmapheresis of dogs had no effect on the concentration or rate of disappearance of unconjugated radioactivity following the injection of C^{14}-estrone, but did produce an increase in the level of the conjugated fraction. The reason for this dichotomy is not known and exemplifies the need for more detailed knowledge of the binding of conjugates than previous experiments have afforded (1, 8). The greater urinary excretion of radioactivity following plasmapheresis is thought to be a reflection of the higher plasma levels of the conjugated steroids rather than an effect of the lowered protein concentration, since the rate of disappearance of the conjugated radioactivity was nearly the same as that before plasmapheresis. This would be consonant with the high binding capacity of albumin for the estrogen conjugates (8). Even at the low concentration of plasma protein attained following plasmapheresis, there were probably several grams of albumin left in the circulation.

Previously reported studies from our laboratory indicated a possible relationship between the binding of a steroid to human serum albumin and its excretion in the bile of human subjects (9). Those steroids which are bound avidly to serum albumin (estrogens, progesterone) are primarily excreted in the bile, while those steroids which are weakly bound by human serum albumin (cortisol, 11β-hydroxyandrostenedione) are not excreted in the bile to any great extent. If the binding to dog serum albumin bears a similar relationship between the steroids and the excretion in the bile, the plasmapheresis and the resultant lack of serum albumin could conceivably result in decreased biliary excretion of the radioactive metabolites of C^{14}-estrone. It should be pointed out that plasmapheresis may remove factors other than serum proteins from the plasma of the dogs which may be necessary for the normal excretion of the metabolites of estrone in the bile. Thus, the changes in distribution may be related more to these factors than to the plasma proteins.

Even though the period of study following injection of the steroid was very brief (20 minutes), it can be seen that in the control rats 80–89 per cent of the radioactivity was already present in the liver and gastrointestinal tract, with 43–71 per cent in the latter. Valcourt *et al.* (6) have shown that following intramuscular or intragastric administration of C^{14}-estrone to rats 70% of the radioactivity appeared in the stools in 4–5 days. Following cannulation of the bile duct, 85% of the radioactivity appeared in the bile, with only negligible amounts in the urine or feces. Studies in our laboratory have shown that following intravenous injection of C^{14}-estrone into rats with bile ducts cannulated, over 90 per cent of the radioactivity is recovered in the bile within one hour (7). The data of the present study also indicate rapid excretion of the radioactivity into the gastrointestinal tract, probably via the bile. The liver contained considerable amounts of radioactivity and, on the basis of the studies mentioned previously (6, 7), there is reason to believe that these metabolites were destined to be excreted in the bile. The endocrine glands examined contained a low level of radioactivity, as did the bladder and its contents of urine, when compared to that of the liver. It is interesting to note that the fat contained more radioactivity per gram of tissue (approximately 500 counts per minute) than the muscle (250 counts per minute). Negligible amounts of radioactivity were present in the blood, most of it appearing in unconjugated fraction of the plasma or associated with the red blood cells.

Since the administration of dextran, and also probably PVP-Macrose, in the rat results in adverse effects on the small blood vessels and other systems (4, 5), it is difficult to interpret the results of plasmapheresis. The presence of considerable radioactivity in the lungs of rats given dextran following plasmapheresis, and in some rats following PVP-Macrose, may be related to pooling, extravascular extravasation or trapping of the C^{14}-estrone by the reticuloendothelial system of the lung. The abnormalities in the distribution of the radioactivity following C^{14}-estrone injection in rats given dextran or PVP may be related not only to the adverse effect of the plasma substitutes, but also to the lack of plasma proteins. Thus, dextran is known to adversely affect the vascular system of the rat (4, 5) and the abnormal distributions of the radioactivity of C^{14}-estrone may be accounted for on the basis of a shock-like state in the rats given dextran. This explanation does not hold true, however, for the rats given PVP. The reasons for the abnormal distribution of the radioactivity in these rats remain unknown.

It has been previously demonstrated that in the rat the biliary route serves as the major pathway for the excretion of steroid metabolites (6, 7, 10–14). Of all the steroids studied, C^{14}-estrone would seem to be the steroid most quantitatively excreted in the bile of the rat. The present studies indicate that the presence of plasma proteins plays an important role in the excretion of these metabolites in the bile of the rat and that in the absence of such proteins, the distribution of the injected radioactive C^{14}-estrone is modified with resultant decreased excretion of its metabolites in the bile.

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THE EFFECT OF ESTROGENS ON BONE PHOSPHATE ACCRETION, RESORPTION, AND EXCHANGE IN MICE

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ABSTRACT

The effect of estrone on the bone phosphate accretion rate, the size of the exchangeable fraction and resorption has been measured. The estrogen treatment enhanced both accretive and resorptive mechanisms associated with the non-exchangeable bone phosphate while there was little effect upon the size of the exchangeable phosphorus pool. The turnover time of stable bone mineral was reduced from 18.5 to 13.0 days by the treatment. An assay procedure in which an "accretion index" was calculated showed the relative potencies of estrone, estradiol benzoate and diethylstilbestrol to be in the ratio of 9:2:1, respectively.

IT IS an established fact that estrogens may modify bone growth and mineral deposition (1), but the extent to which these hormones effect such changes has not always been quantitatively elucidated. Recently Bauer *et al.* (2, 3) have described an analytical approach that makes it possible to measure separately the apparent accretive and resorptive mechanisms that exist simultaneously in bone. We have applied the formulae of Bauer *et al.* to a study of the effect of estrogen on bone phosphate accretion and resorption in mice. In addition, we have used an "accretion index" to define the relative potency of three estrogenic materials in terms of their ability to modify bone metabolism.

METHODS

Young, adult male mice fed a commercial laboratory chow were divided into 10 groups of 8 animals each. Five groups received 0.8 mg./kg. estrone in corn oil daily for 22 days, while the others received oil alone. At intervals of 4, 24, 48, 96 and 144 hours before sacrifice a treated and a control group were given subcutaneously 2 microcuries of P^{32} as sodium phosphate. All animals were sacrificed on the 23rd day and plasma and left tibiae were sampled. Plasma inorganic phosphate was estimated in the manner described by Bauer and Carlsson (2) using the colorimetic method of King (4). Aliquots of the acid filtrate of plasma were mounted directly on lens paper supported by copper planchets and the radioactivity measured with an end-window geiger tube. Tibia samples were autoclaved to facilitate removal of flesh and after cleaning were dried overnight at 110° C. The bones were weighed, dissolved in 1 ml. nitric acid and then 0.2 ml. perchloric acid was added to clarify the solution. Water was added to make 5 ml. and aliquots were mounted for radioactivity measurement as described above.

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To measure the effect of estrone on the apparent turnover rate of bone phosphate 2 groups of 16 male mice each were injected subcutaneously with 2 microcuries of radioactive sodium phosphate. The treated group then received daily 0.8 mg./kg. of estrone in corn oil; the control received the vehicle alone. Half the animals in each group were sacrificed after 7 days of treatment and the remainder after 14 days. Tibiae were analyzed for P^{32} as described above.

A simplification of the experimental approach described above provided a convenient assay procedure by which a comparison could be made of the effects of various estrogenic materials upon the accretive phase of bone metabolism. Estrone, estradiol benzoate, and diethylstilbestrol were administered daily in oil for a period of nine days to groups of 10 animals each. On the 5th day after the start of the treatment all animals including a control group received two microcuries of P^{32}; the mice were sacrified on the 10th day and plasma and tibiae were sampled. The measurements of plasma phosphate specific activity and of bone P^{32} were carried out as described above.

RESULTS AND DISCUSSION

Bone Phosphate Accretion, Exchange and Resportion. Table 1 illustrates the bone uptake of P^{32} and the specific activity of plasma inorganic phosphate as well as plasma inorganic phosphate concentration. Although there is a variation in plasma phosphate level between groups the average of the 40 treated mice is nearly identical to that of the 40 control animals.

TABLE 1. DISTRIBUTION OF P^{32} IN TIBIA AND PLASMA OF MICE TREATED WITH ESTRONE

Time (hrs.)	Plasma phosphate (μg./ml.)		Plasma specific activity* (% P^{32}/μg. P × 10^3)		Tibia P^{32} (% P^{32}/gm.)	
	Control	Estrone	Control	Estrone	Control	Estrone
4	84.8	57.0	2.86	2.90	8.69 ± .39	10.5 ± .7**
24	58.6	51.7	2.04	1.74	9.47 ± .48	12.1 ± .7***
48	53.2	71.4	1.49	1.52	12.02 ± 1.14	12.2 ± .5
96	67.6	79.4	.769	.886	8.87 ± .32	11.6 ± .7***
144	71.5	75.5	.504	.577	8.73 ± .41	11.2 ± .4**
Mean	67.1	67.0	—	—	—	—

* Plasma specific activity is per cent P^{32} recovered per microgram plasma phosphate.
** $P < .05$.
*** $P < .01$; unstarred value is not significantly different from control.

For the calculation of the accretion rates, equation (7) of Bauer *et al.* (3) has been slightly modified.

$$\frac{P_{O4} - ATS_{M4}}{P_{O24} - ATS_{M24}} = \frac{S_4}{S_{24}} \tag{1}$$

where: P_O is the per cent administered P^{32} recovered per gram bone at the time indicated (subscript); A is the accretion rate; T is the time after the administration of the isotope; S_M is the mean specific activity[1] of plasma

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[1] Specific activity is defined as the per cent administered P^{32} recovered/μg. plasma phosphate.

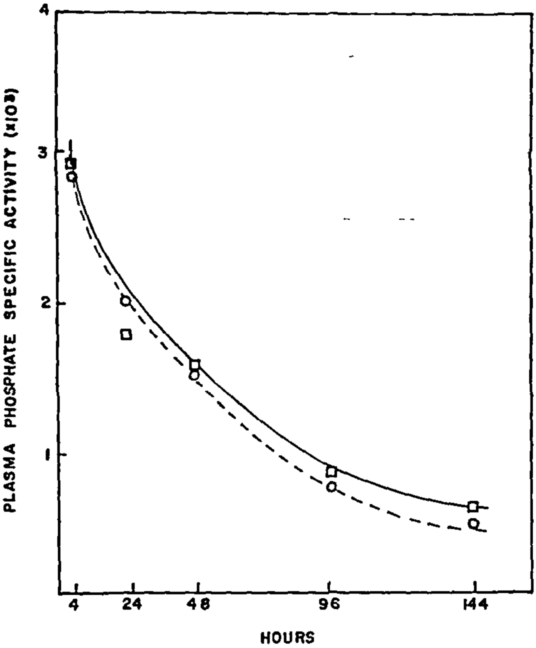

FIG. 1. Plasma specific activity-time curves. The abscissa indicates elapsed time after administration of isotope; the ordinate indicates specific activity defined as per cent P^{32} recovered per µg. plasma phosphate. Symbols: open circle, control; open square, estrone 0.8 mg./kg.

phosphate during this time interval; and S is the specific activity[1] of plasma phosphate at any given time. The integrated function, TS_M, was measured planimetrically from the specific activity-time curves for the plasma phosphate of the control and treated animals (Fig. 1), and P_O and S were taken from Table 1. Solving for A, the accretion rate[2], resulted in a value appreciably higher in the estrone treated animals than in the controls (Table 2). Since the value P_O in the estrone treated animals was in almost every case significantly different from that for the control animals, we assumed this difference in accretion rate to be statistically significant. To calculate E, the amount of P in the exchangeable fraction in bone, we have modified equation (4:1) of Bauer *et al.* (3) to read:

$$P_{O24} = ES_{24} + ATS_{M24} \tag{2}$$

where: E is the amount of P in the exchangeable fraction of bone; and the other symbols are as defined above. As shown in Table 2 the size of the exchangeable fraction of bone P was essentially unchanged by the estrone treatment. From these values for the rate of accretion of bone P and the size of the exchangeable P pool of bone we have calculated: P_A, the per cent administered P^{32} incorporated into the non-exchangeable fraction of bone through accretion,

$$P_A = ATS_M; \tag{3}$$

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[2] During the interval 0–24 hours after isotope administration Bauer, *et al.* (3) demonstrated that resorptive mechanisms do not affect P^{32} levels in bone and therefore may be ignored in the calculation of "A" and "E" during this time interval.

TABLE 2. THE ACCRETION RATE AND THE EXCHANGEABLE FRACTION IN TIBIA OF MICE TREATED WITH ESTRONE

Treatment	Mean body wt. (gm.)	Accretion rate, "A" (μg. P/gm./hr.)	Exchangeable fraction, "E" (μg. P/gm.)
Control	32	82.5	2.56×10^3
Estrone, 0.8 mg./kg.	34	112.0	2.96×10^3

P_E, the per cent administered P^{32} present in the exchangeable fraction of bone,

$$P_E = ES; \tag{4}$$

and P_R, the per cent administered P^{32} removed through resorption,

$$P_R = P_A + P_E - P_O. \tag{5}$$

The bone fraction-time curves illustrating these values for control and estrone treated animals are shown in Fig. 2. The control pattern shown in this report is not unlike that presented by Bauer, *et al.* (2) for the rat. From the reports of these workers (2, 3) we have estimated a tibia shaft accretion rate of approximately 45 μg. P/hr./gm. in a 250 gm. rat, which is of same order of magnitude as our value of 83 μg. P/hr./gm. for the whole tibia of the mouse. It must be remembered that the accumulation of isotope in the shaft proceeds at a slower rate than in the ends of the long bones (3).

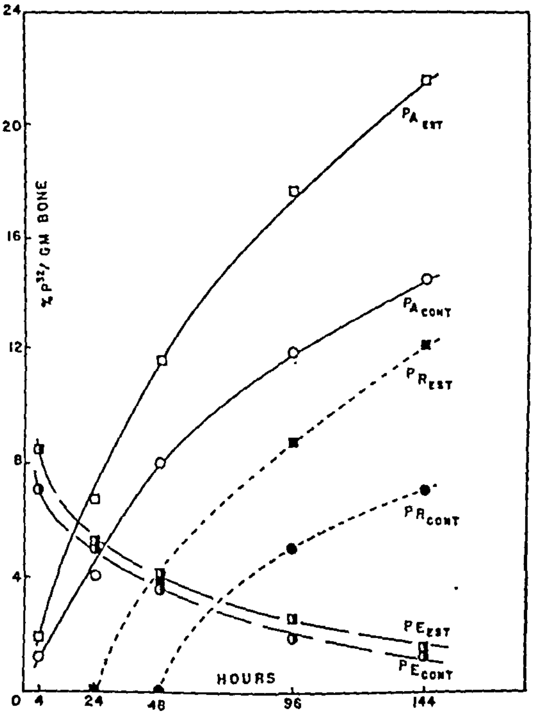

FIG. 2. Bone fraction-time curves. The abscissa indicates elapsed time after administration of isotope; the ordinate indicates per cent isotope calculated in bone fraction. Symbols: P_A, accretive incorporation of isotope into non-exchangeable fraction of bone; P_R, resorptive removal of isotope from bone; P_E, isotope present in exchangeable fraction of bone. Subscripts CONT and EST designate control and estrone treated groups, respectively.

It is evident that, whereas the amount of residual P^{32} in the exchangeable fraction (P_E) was only slightly increased by the treatment, both accretion and resorption mechanisms were stimulated to greatly increased activity by estrone.

Bone Phosphate Turnover. To confirm directly the evidence that bone phosphate turnover was enhanced by estrone treatment we have calculated the turnover time from the rate of disappearance of bone phosphate during the interval 7–14 days after isotope administration (Fig. 3). At this time interval the amount of bone P^{32} contributed by the exchangeable fraction is very small (Fig. 2), and the observed decrease in bone P^{32} can be attributed to resorptive mechanisms associated with remodeling of the bone structure. Calculation of the turnover time of bone phosphate according to the method of Zilversmit *et al.* (8) resulted in a value of 18.5 days for the control animals while the estrone treated mice exhibited a turnover time of 13.0 days.

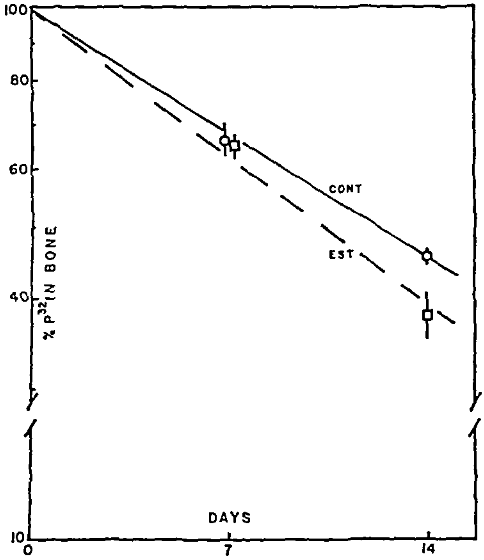

FIG. 3. Bone phosphate disappearance curves. The abscissa indicates the elapsed time after isotope injection; the ordinate shows the per cent P^{32} remaining in bone. The curves have been normalized so that the intercept at time 0 = 100%. The normalizing factor = 2.0. The vertical lines indicate the standard errors.

The evidence presented in this report indicates estrone augmented the turnover of bone phosphate in mice, both accretive and resportive mechanisms being stimulated to higher levels of activity than were observed in the control animals. Since Edgren and Calhoun (5) in similar experiments have shown estrone to induce an increase in bone density, we must conclude that to effect such a net increase in bone mineral phosphate the hormone must necessarily have enhanced bone mineralization more profoundly than bone remodeling.

Bone Accretion Index. To effect a quantitative comparison among various estrogenic materials an assay based on equation (3) described in the

TABLE 3. THE EFFECT OF ESTROGENS ON THE BONE ACCRETION INDICES OF MALE MICE

Experiment	Group	Compound	Dose (mg./kg.)	Plasma specific activity "S"†	Bone P^{32} "Po"†	Accretion index "AI"†	Relative potency as determined by:	
							Accretion index	Bone density (5)
1	1	Control	—	.570	19.0	33.4 ± 4.0		
	2	Estrone	0.1	.444	17.1*	38.6 ± 1.7	—	—
	3	Estrone	0.4	.390	17.1*	43.8 ± 3.3	—	—
	4	Estrone	0.8	.326**	17.8	54.6 ± 4.4**	9.0	7.3
	5	Estradiol Benzoate	3.0	.410	21.8	53.1 ± 4.7**	2.1	2.7
	6	Diethylstilbestrol	7.0	.355*	17.0*	47.8 ± 2.2**	1.0	1.0
2	7	Control	—	.550	23.3	42.4 ± 3.8		
	8	Estrone	0.5	.470	28.1*	59.7 ± 3.8*	—	—
	9	Estrone	1.0	.473	29.7	62.8 ± 5.5**	7.9	7.3
	10	Diethylstilbestrol	7.0	.542	30.2**	55.7 ± 4.8*	1.0	1.0
	11	SC-6924***	50.0	.566	25.8	45.6 ± 1.7	0	0.01

† Symbols are defined in the text, equation (6).
* $P < .05$; unstarred values are not significantly different from controls.
** $P < .01$.
*** SC-6924, Manvene, is 3 methoxy 16 methyl, 1, 3, 5(10) estratriene 16β 17β diol, and is an estrone derivative that profoundly modifies the lipid metabolism of the chick (6) but evidences only weak estrogenic properties.

preceding section has been developed. We have defined the accretion index as:

$$AI = \frac{P_O}{S} \tag{6}$$

where: AI is the accretion index; P_O is the per cent administered P^{32} recovered per gram bone; and S is the specific activity of plasma phosphate at the time of sacrifice. In arriving at this means of quantitatively expressing the effect of an estrogen upon bone metabolism we have ignored the effects of resorption and have assumed the plasma specific activity at $T = 120$ hours to be proportional to the integrated mean S_M. In eliminating consideration of resorption in the calculation we err on the conservative side, since we are, in effect, calculating a function of the *net* accretion rather than one of the total synthetic product. The similarity of shape of the plasma specific activity curves of the control and treated mice (Fig. 1) lends credence to our assumption that the plasma specific actvities at 120 hours are representative of S_M. We have in addition assumed the contribution of the exchangeable fraction, P_E, to the observed bone P^{32} at $T = 120$ hours to be negligible and the curves described in Figure 2 show this assumption to be essentially correct. As can be seen in Table 3 neither the plasma specific activity, S, nor the per cent P^{32} incorporated into bone, P_O, is as reliable a test of the ability of a material to enhance mineral deposition as is the accretion index. In two experiments using diethylstilbestrol, estradiol benzoate, and estrone a comparison of our potency estimates with those of Edgren and Calhoun (5), derived from bone density studies exhibited excellent agreement of the two methods.

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EFFECT OF CORTISONE ON GROWTH OF CHICK EMBRYOS DURING EARLY EMBRYOGENESIS[1]

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ABSTRACT

Cortisone acetate was administered on the initial day of incubation and at two or three day intervals thereafter to Cornish cross-White Hampshire chick embryos. The weight and length measurements were compared with controls on the fourth, seventh and tenth incubation days. A growth retarding effect of cortisone was demonstrated, which was more strikingly manifested in the alteration of the average length of the embryo in the earliest period, and in the alteration in average weight in the later periods. With this demonstration of growth retardation as early as the fourth day of incubation, it would appear unlikely that this effect is due to a suppression of somatotropin secretion by the hypophysis, since the latter is not fully differentiated prior to the tenth day. It would appear more likely that this effect represents a direct inhibition of protein synthesis and growth by cortisone.

RETARDATION of the growth rate and changes in the anatomy of the developing chick embryo following the administration of adrenal cortical extract (7), DCA (11), and cortisone acetate (3, 6, 13) have been reported. From these observations it would appear that there is a symmetrical retardation of growth. Karnosfsky *et al.* (6), in particular, have further reported that cortisone does not affect the growth rate until after the eighth day of development, and that the hormonal effect is diminished after the tenth day. They have postulated, therefore, that cortisone may interfere with some new substrate which normally appears during the mid-period of embryogenesis (eighth to twelfth days), coincident with the development of many new functions, including the peak development of the mesonephros.

Siegel *et al.* (13) have considered this cortisone effect as mediated through an inhibition of the pituitary, based primarily on the observation of Fugo (5) that hypophysectomy of the chick embryo has no appreciable effect on its growth and development until after the tenth day of incubation, and by analogy from the observations on postnatal animals of various species (2, 4, 12, 14), which show cortisone effects having a hypophyseal depend-

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ency. Furthermore, Allbright (1) has considered the well-known suppression of protein synthesis by cortisone an "anti-anabolic" rather than a direct catabolic effect, and Li (8) is of the opinion that this effect is one of an antagonism to hypophyseal growth hormone. On the other hand, Buno and Goyena (2) have shown a direct *in vitro* inhibition of the growth of chick embryo femur by cortisone. The present experiments compare the growth retardation of cortisone on the chick embryo prior to day 4, before the development of an intrinsic functional endocrine system, with that obtained by days 7 and 10 when there is doubtful intrinsic endocrine activity.

MATERIALS AND METHODS

Cornish-cross, White Hampshire chick eggs were drilled with a sterile teasing needle and injected chorioallantoically with the various solutions and suspensions noted below, using a 1 ml. syringe. In the first experiment the embryos received injections on the day of incubation, and on the third, sixth and ninth days thereafter. In the second experiment injections were made on the day of incubation, and on the second, fourth and sixth days thereafter. And in the third experiment the embryos were injected on the day of incubation and on the second day thereafter. In the first two experiments the embryos were examined on the day following the last injection, and in the third experiment on the fourth incubation day. In each experiment, all groups were injected at the same time and incubated simultaneously.

TABLE 1. INJECTED ON DAYS 0, 3, 6 AND 9: EXAMINED ON DAY 10

Experiment	Dose	Survival rate	Average weight	Average length
Cortisone Acetate	1 mg. in 0.2 ml.	13/18	1.27 ± .14$_1$* gm. (1.05–1.55)**	27.6 ± 1.5* mm. (25.0–30.0)**
Cholesterol	1 mg. in 0.2 ml.	10/18	1.64 ± .09 gm. (1.45–1.75)	30.2 ± 2.8 mm. (21.0–30.5)
Sodium Chloride	180 mg. in 0.2 ml.	12/18	2.93 ± .13 gm. (2.65–3.20)	32.8 ± 0.5 mm. (32.0–33.5)
Sham Operated		15/18	2.73 ± .14 gm. (2.50–3.0)	34.6 ± 0.5 mm. (33.5–35.5)

* Standard deviation.
** Range of variation.

One group in each experiment received 1 mg. of cortisone acetate in 0.2 ml. of distilled water per injection; another group in each experiment received 0.2 ml. of 0.9% saline per injection, and a third group was sham operated. A cholesterol suspension (1 mg. in 0.2 ml. distilled water) was used as an additional control in the first experiment, but this was replaced by an aqueous solution of sodium desoxycholate in experiments 2 and 3, because of the latter's closer chemical identity with cortisone and because the insolubility of cholesterol presented difficulties in maintaining a uniform suspension. Thus each experiment consisted of 3 control groups and a cortisone test group.

At the completion of the incubation period, the embryos were separated from all extraembryonic materials, blotted thoroughly and weighed individually. They were also then individually measured from crown to extended toe. The data thus obtained were averaged and standard deviations determined, as shown in Tables 1, 2, and 3.

RESULTS

The data in the first experiment (Table 1) show that the average weight of the cortisone injected embryos is less than one-half that of either the saline or sham operated controls; the cortisone group also shows a significantly lower average weight than the cholesterol injected embryos, but the difference is not as great. As to length measurements, the difference between the cortisone and cholesterol groups is not significant, but the saline and sham operated groups each show an average length significantly greater than the other two.

TABLE 2. INJECTIONS ON DAYS 0, 2, 4 AND 6: EXAMINED ON DAY 7

Experiment	Dose	Survival rate	Average weight	Average length
Cortisone Acetate	1 mg. in 2 ml.	27/40	0.65 ± .04* gm. (.65- .78)**	15.5 ±0.5* mm. (15.0–16.5)**
Sodium Desoxycholate	1 mg. in 2 ml.	21/26	1.41 ± .10 gm. (1.25–1.60)	22.6 ±0.7 mm. (21.5–23.5)
Sodium Chloride	180 mg. in 2 ml.	14/20	1.56 ± .05 gm. (1.50–1.65)	19.9 ±0.9 mm. (18.0–21.5)
Sham Operated		36/40	1.27 ± .05 gm. (1.20–1.35)	21.1 ±0.5 mm. (20.5–22.0)

* Standard deviation.
** Range of variation.

The weight data in the second experiment (Table 2) also show that the average weight of the cortisone injected embryos is less than one-half that of either the desoxycholate or saline injected controls, and only slightly more than one-half that of the sham operated group. While the data on length measurements are not as striking, they also show that, on the average, cortisone injected embryos are significantly shorter than embryos in any of the three control groups.

Whereas, in the first two experiments the growth retardation in the cortisone injected embryos is more strikingly manifested in changes in body weight than in body length, in the third experiment the opposite appears to be the case. While, on the average, the weight is lower in the cortisone injected embryos than in the three control groups, the difference between the embryos receiving cortisone and those receiving sodium desoxycholate or saline is not significant. On the other hand, the average length is significantly less in the cortisone injected embryos than in the three control groups. The fact that sodium desoxycholate and sodium chloride produce some growth retardation, as manifested by both weight and length measurements, suggests that the mere introduction of fluid into very young chick embryos may have some effect in slowing growth processes.

TABLE 3. INJECTED ON DAYS 0 AND 2: EXAMINED ON DAY 4

Experiment	Dose	Survival rate	Average weight	Average length
Cortisone Acetate	1 mg. in 0.2 ml.	29/39	.023 ± .002* gm. (.021–.025)**	.087 ± .002* mm. (.082–.090)**
Sodium Desoxycholate	1 mg. in 0.2 ml.	27/39	.031 ± .006 gm. (.020– .040)	.100 ± .009 mm. (.085–1.250)
Sodium Chloride	180 mg. in 0.2 ml.	18/39	.030 ± .006 gm. (.022– .038)	.108 ± .008 mm. (.100– .125)
Sham Operated		20/39	.038 ± .004 gm. (.032– .040)	.126 ± .005 mm. (.115– .135)

* *Standard deviation.*
** Range of variation.

DISCUSSION

These results show that when cortisone is administered on the initial day of incubation and at 2 or 3 day intervals thereafter, there is a retardation of growth which can be detected as early as the fourth day of incubation, and is also demonstrable on the seventh and tenth incubation days. The use of non-hormonal steroids as one of the controls serves to indicate that this growth retardation is, in all likelihood, a specific hormonal effect. Furthermore, the data show that, contrary to the report of Karnofsky *et al.* (6), this effect by cortisone can be demonstrated before the eighth incubation day. This growth retardation is more strikingly manifested in the alteration of the average length of the embryo in the earliest period and in the alteration in the average weight in the later periods. Although these experiments do not provide an explanation of this difference, it may be related to the fact that during the earliest periods of embryogenesis processes of differentiation are predominant, while at later periods processes of growth become more pronounced. The weight differences observed in these experiments cannot be accounted for on the basis of retention of tissue fluids, since the well-known property of adrenal corticoids of causing a retention of water and electrolytes would tend to increase, rather than to decrease, the weight of embryos receiving cortisone.

As has been pointed out, the suppression of protein synthesis by cortisone is believed by Allbright (1) to be an "anti-anabolic" effect, the mechanism of which Li (8) would apparently consider to be that of an antagonism to hypophyseal Growth Hormone. On the other hand, the observation of Buno and Goyena (2) that cortisone can cause an *in vitro* inhibition of growth of chick embryo femur may be taken to indicate that the latter hormone is also capable of inhibiting growth which may not be somatotropin dependent. The present observations appear further to support such a conclusion, since it is unlikely that hypophyseal Growth Hormone is active as early as the fourth day of embryogenesis of the chick. Rahn (9) has reported that Rathke's pouch becomes metamorphosed into a circumscribed mass of solid cell strands within a mesenchymatous sheath by about the fifth day. The entire buccal portion of the hypophysis shows an affinity for basic dyes on the eighth day, but the basophilic and acidophilic cells do not differentiate as such until the tenth day. It would thus appear unlikely that somatotropin secretion occurs before the tenth day, and thus all three of our experiments may be interpreted as demonstrating a growth retardation not dependent upon somatotropin suppression, possibly even a direct inhibition of protein synthesis and growth by cortisone.

Nevertheless, some caution as regards the latter conclusion is in order. Rahn and Drager (10) have pointed out that the time at which hormones are produced by the hypophysis does not necessarily correspond to the time at which staining differences appear; the melanophore-dispersing hormone of the hypophysis is first detected on the fifth day, although definite cellular types do not appear in the gland until the tenth day. However the observation of Fugo (5) that hypophysectomy of the chick embryo has no appreciable effect on its growth and development until after the tenth day of incubation makes it unlikely that endogenous somatoropin is secreted in significant amounts prior to that time. Nevertheless, it is, as yet, not possible to exclude the presence in the yolk of some growth stimulant of maternal origin to which cortisone may be antagonistic. Thus, while the present data indicate that the growth retarding effect of cortisone during the first 10 days of embryogenesis is, in all likelihood, not a manifestation of endogenous somatotropin inhibition, further investigation is necessary to determine whether or not this is a direct effect of cortisone.

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STUDIES OF THE SUPPRESSION OF AMINONITRILE LESIONS IN RATS BY THYROXINE ANALOGUES[1]

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ABSTRACT

In contrast with the striking suppressing effects of triiodothyronine and thyroxine on the lesions produced by aminoacetonitrile in rats, the lesions produced by β-aminopropionitrile were only slightly suppressed by these hormones.

The activity of eight thyroxine analogues in the suppression of the aminoacetonitrile lesions in rats was found to be far below that of triiodothyronine.

Corticosterone and cortisone suppressed only slightly the lesions produced by aminoacetonitrile in rats.

AMINONITRILES administered to vertebrates during a certain period of their growth cause extensive derangement of the mesodermal tissues, including skeletal deformities (1, 2, 3). Of the many aminonitriles tested we have found only three which produce the mesodermal lesions: aminoacetonitrile (AAN) (4), methyleneaminoacetonitrile (MAAN) (5), and β-aminopropionitrile (BAPN) (4). The last compound is found in the form of β-(γ-L-glutamylamino) propionitrile in the seeds of certain legumes of the Lathyrus family (6). The lesions produced by these three aminonitriles appear histologically to be similar, although in the rat AAN and MAAN are about four times more active than BAPN (5). However, certain differences have been observed in the action of these compounds. For example, frog larvae are responsive to very low concentrations of BAPN but are not affected by AAN (3). Dissecting aneurysm of the aorta is frequently observed in weanling rats fed BAPN but rarely in rats fed AAN or MAAN. The epiphyseal plates of rats treated with AAN or MAAN are wider but have fewer rents than the plates of rats fed BAPN. Kyphoscoliosis develops very frequently in rats treated with BAPN and rarely in rats treated with AAN. Dasler has found that semicarbazide and mercaptoethylamine produce lesions in rats similar to the BAPN lesions (7, 8).

Lately we have described suppression of the lesions produced by AAN with low doses of L-3:5:3′-triiodothyronine (TRI) (9); the first series of experiments reported here has shown that TRI has little suppressing effect upon the lesions produced by BAPN.

Triiodothyropropionic acid and tetraiodothyropropionic acid have been

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found to be much more active than TRI in accelerating the metamorphosis of tadpoles of *Rana esculenta* (10). In the second series of experiments here reported, none of a number of analogues of TRI and thyroxine tested was found to be as active as TRI in the suppression of the AAN lesions.

Selye and Bois (11) have described the suppressing effect of cortisol acetate upon the lesions produced by AAN in rats. Our results with the administration of corticosterone and cortisone acetate[2] in rats treated with aminoacetonitrile are here reported.

I. *Effect of Thyroxine and Triiodothyronine on the Lesions Produced by β-aminopropionitrile in Rats*

In the first experiment (Table 1) weanling male Sprague-Dawley rats weighing 42 to 50 gm. were fed *ad libitum* ground Purina chow containing 0.18% β-aminopropionitrile fumarate.[3] The fumarate salt of the BAPN is a solid and easier to use in feeding experiments than the liquid BAPN. The animals were housed in cages in groups of five. Various doses of thyroxine (Tx), TRI, 3:5:3′:5′-tetraiodothyropropionic acid, and 3:5:3′-triiodothyropropionic acid were injected subcutaneously daily in slightly alkaline solutions. Two groups of rats served as controls: one group was fed

TABLE 1. EFFECT OF L-THYROXINE (TX), L-TRIIODOTHYRONINE (TRI) AND ANALOGUES IN PREVENTION OF SKELETAL LESIONS IN RATS GIVEN PURINA CHOW CONTAINING 0.18% OF β-AMINOPROPIONITRILE[1] (BAPN)

Treatment[2] (μg./day/rat)	Average		Severity of lesions
	Starting weight (gm.)	Final weight (gm.)	
Purina only[3]	49	157	0
Purina + BAPN[3]	49	126	3+
Tx			
50	48	129	3+
125	48	118	2+
TRI			
2[4]	49	127	3+
9[5]	49	97	2+
3:5:3′:5′-Tetraiodothyropropionic acid			
2	48	124	3+
9	48	122	3+
3:5:3′-Triiodothyropropionic acid			
2	48	123	3+
9	44	120	3+

5 rats were used to test each dose. Duration of experiment, 18 days.

[1] Given as salt of fumerate.

[2] Tx, TRI and analogues were administered in slightly alkaline solutions and injected subcutaneously.

[3] 0.2 ml. normal saline injected daily.

[4] One rat died on the 17th day of the experiment of dissecting aneurysm of the aorta.

[5] Two rats died on the 17th day of experiment: one had a dissecting aneurysm of the aorta; no cause of death was found for the other.

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[2] Cortone, Merck & Co.

[3] Courtesy of Abbott's Laboratories, Chicago, Illinois.

Purina chow alone; another group received the BAPN diet alone. The rats in the control groups received 0.2 ml. normal saline injected daily. The animals were weighed every four days. Duration of the experiment was 18 days.

One rat of the group receiving BAPN diet plus TRI (2 μg. daily) died of dissecting aneurysm of the aorta on the seventeenth day of the experiment. On the same day two rats died of the group receiving BAPN diet plus TRI (9 μg. daily). At the post mortem examination one rat had a massive hemothorax due to a dissecting aneurysm of the aorta; no cause of death was found for the other animal.

In the second experiment (Table 2) the rats were fed for 18 days Purina chow containing 35% finely ground *Lathyrus odoratus* meal. Mild skeletal lesions were observed in the roentgenograms taken on the 18th day of the experiment. A diet containing 50% *Lathyrus odoratus* meal was then administered for the following 12 days. Various doses of Tx and TRI were injected daily in slightly alkaline solutions. Five groups of rats fed Purina chow alone receiving similar doses of Tx and TRI served as controls. The rats of two other control groups were injected daily with 0.2 ml. normal saline. One of these groups was fed Purina chow alone and the other the *Lathyrus odoratus* diet. Duration of the experiment was 30 days. One rat receiving the *Lathyrus odoratus* diet and normal saline died of dissecting

Table 2. Effect of l-thyroxine (Tx) and l-triiodothyronine (TRI) in prevention of skeletal lesions in rats given Purina chow containing Lathyrus odoratus meal (35% for first 18 days and 10% for following 12 days)

Treatment (μg./day/rat)	Average		Severity of lesions
	Starting weight (gm.)	Final weight (gm.)	
(A) Purina only[1]	55	227	0
Tx 50	54	230	0
Tx 100	54	245	0
TRI 2	54	233	0
TRI 6	54	239	0
TRI 9	54	192	0
(B) Purina + *L. odaratus*[1,2]	55	136	4+
Tx 50	54	181	3+
Tx 100[3]	54	147	2+
TRI 2[4]	54	178	3+
TRI 6[5]	54	162	3+
TRI 9[6]	54	141	2+

5 rats were used to test each dose in the experimental and in the control groups. Duration of experiment, 30 days.

[1] 0.2 ml. normal saline injected daily.

[2] One rat died on the 14th day of the experiment of dissecting aneurysm of the aorta.

[3] Two rats died on the 13th day of the experiment, cause undetermined. One rat died on the 21st day of the experiment of dissecting aneurysm of the aorta.

[4] One rat died on the 22nd day of the experiment of dissecting aneurysm of the aorta.

[5] Two rats died on the 14th day of the experiment, cause undetermined.

[6] Two rats died of dissecting aneurysm of the aorta, one on the 20th and the other on the 24th day of the experiment.

aneurysm of the aorta on the 14th day of the experiment. Four rats of the experimental groups receiving Tx or TRI died of dissecting aneurysm of the aorta between the 20th and 24th days of the experiment. Four rats of the same groups died between the 13th and 14th days of the experiment; the post mortem examination did not reveal any abnormality to explain the cause of death in these rats.

At termination of each experiment the animals were sacrificed with ether and roentgenograms were made. At the post mortem examination the knees, including lower half of femur and upper half of tibia, were excised and after fixation in 10 per cent neutralized formalin were decalcified in 6 per cent aqueous solution of sulfosalicylic acid. Sagittal sections were stained with hematoxylin and eosin. The roentgenograms were evaluated for a) width of the epiphyseal plates adjoining the knee, b) amount of periosteal new bone formation, c) severity of the kyphoscoliosis, d) width of the ilium, and e) severity of the osteoporosis. The approximate estimate of severity of the lesions obtained from the roentgenograms was checked against the lesions observed in histological sections. Rats of the same experimental group showed only slight variations in the severity of the skeletal lesions.

In contrast with the striking suppressing effects of Tx and TRI on the skeletal lesions produced by AAN (9), the lesions produced by BAPN were only slightly suppressed by the thyroid hormones. The animals on the highest doses of Tx (125 μg./day) and TRI (9 μg./day) had the least severe lesions. The rats of the control groups receiving TRI (9 μg./day) had epiphyseal plates narrower than the normal animals with fewer cartilage cells, indicating retardation of growth (Fig. 1). However, the epiphyseal plates of the experimental animals on a similar dose of TRI were wider than those of normal animals and rents were apparent in the proliferating zone of the plate. The abnormalities were even more pronounced in rats on the *Lathyrus odoratus* diet which received 2 μg./day of TRI or 100 μg./day of Tx (Fig. 2). The kyphoscoliosis was less severe in the animals receiving TRI 9 μg./day but was not completely prevented, neither were osteoporosis, lesions of the aorta, and bone deformities.

II. *Relative Potencies of Tx and TRI Analogues in the Suppression of AAN Lesions in Rats*

In our quest for a compound which, when administered in non-toxic doses, would arrest the lesions produced by the aminonitriles, we tested a variety of Tx and TRI analogues (Table 3). Groups of six weanling male Sprague-Dawley rats fed Purina chow were injected subcutaneously daily with 6 mg. AAN for the first 8 days of the experiment and 9 mg. AAN for the following 9 days. Tx, TRI, or the analogues were injected during this period in the doses indicated in Table 3.[4] Two groups of rats served as controls: One group was fed Purina chow alone, another group received the AAN injections without thyroid hormones or analogues. Duration of the experiment was 17 days. At the termination of the experiment the animals were sacrificed and roentgenograms were

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[4] The author is indebted to Dr. Robert L. Kroc of the Warner Chilcott Laboratories for kindly providing the analogues used in this experiment.

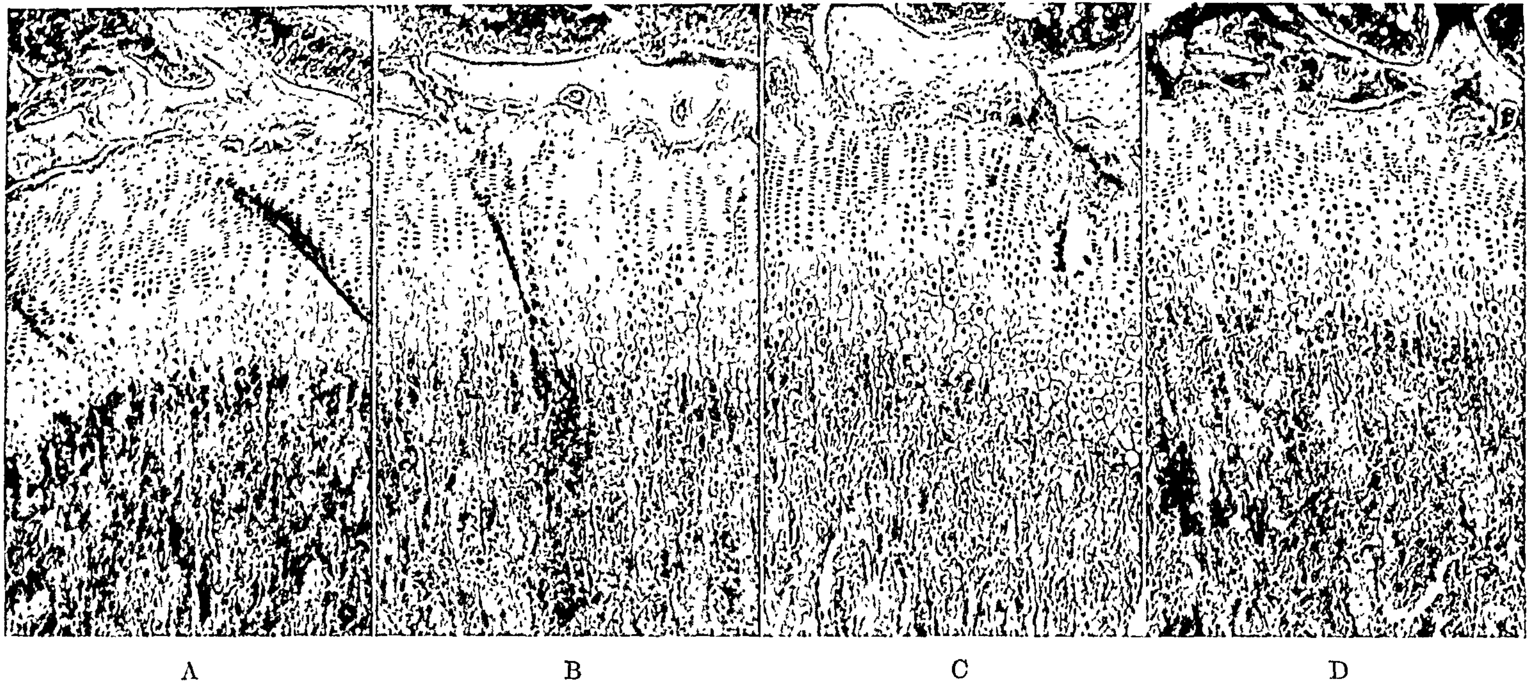

FIG. 1. Proximal epiphyseal plates of the tibia of 56-day-old rats (×40, hematoxylin and eosin stain) treated as follows for 30 days:
A. Normal control.
B. Tx (100 μg. per day) injected subcutaneously.
C. TRI (2 μg. per day) injected subcutaneously.
D. TRI (9 μg. per day) injected subcutaneously.

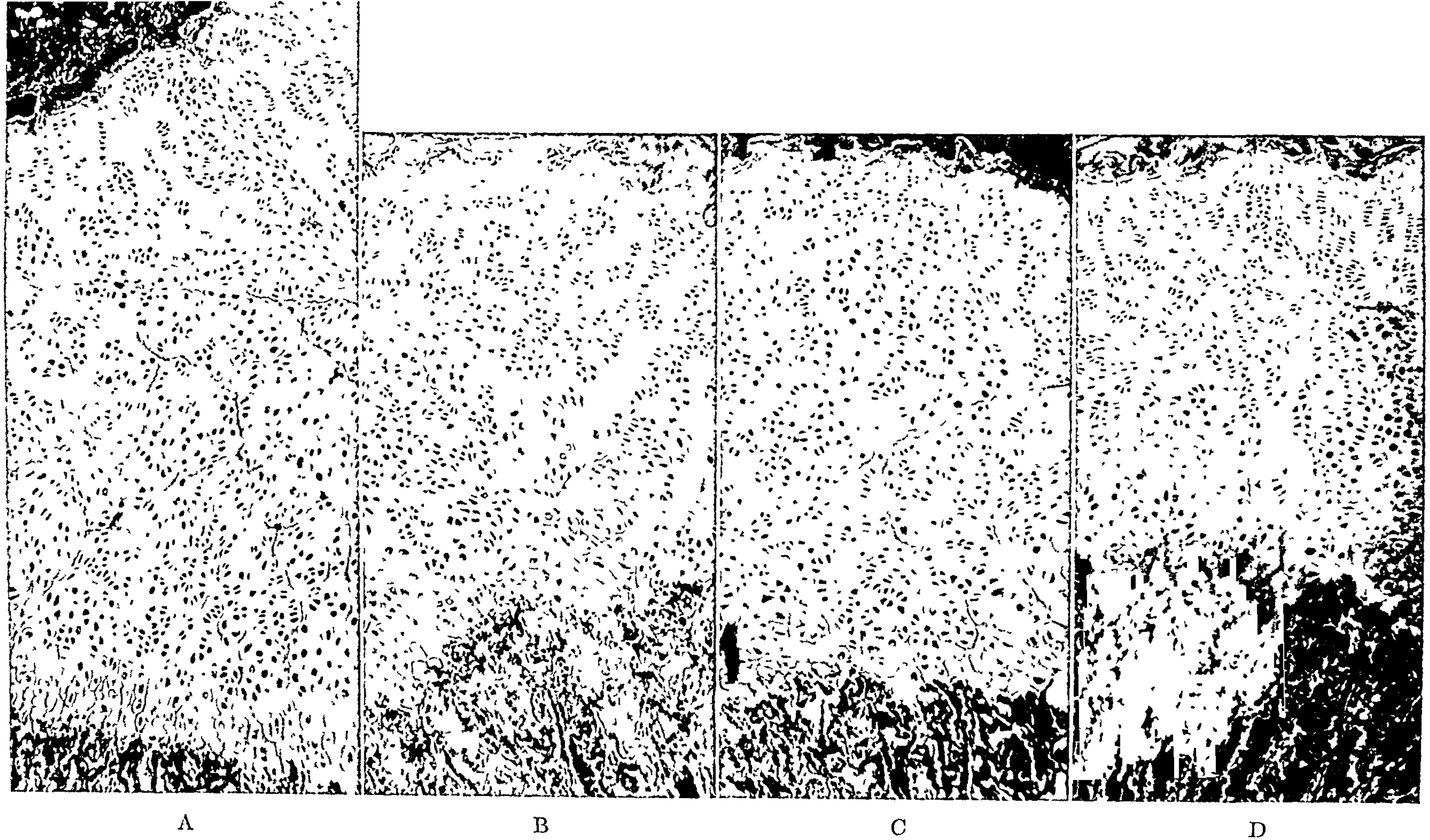

FIG. 2. Proximal epiphyseal plates of the tibia of 56-day-old rats (×40, hematoxylin and eosin stain) treated as follows for 30 days:
A. *Lathyrus odoratus* diet (35% for the first 18 days and 50% for the following 12 days).
B. *L. odoratus* diet plus Tx (100 μg. per day) injected subcutaneously.
C. *L. odoratus* diet plus TRI (2 μg. per day) injected subcutaneously.
D. *L. odoratus* diet plus TRI (9 μg. per day) injected subcutaneously.

made. At postmortem examination no instance of dissecting aneurysm of the aorta was noted in either control or experimental animals. The knees were excised and processed for histological studies as in the previous experiments. An approximate estimate of the severity of lesions obtained from the roentgenograms was checked against the lesions observed in the histological sections.

As in previously reported experiments (9), TRI was estimated to be at least 50 times more active than Tx in the suppression of the lesions produced by AAN. The activity of the analogues tested was far below that of TRI. The compounds 3:5:3′-triiodothyroacetic acid, 3:5:3′-triiodothyropropionic acid, 3:5:3′:5′-tetraiodothyroacetic acid and 3:5:3′5′-tetraiodothyropropionic acid were found to have an activity similar or slightly below that of Tx. The compounds with the iodine atoms in position 3:3′:5′ in the thyronine molecule showed practically no activity in our experiments. Neither 3:3′-diiodothyronine nor the propionic acid analogue showed any significant activity.

TABLE 3. EFFECT OF INJECTED L-TRIIODOTHYRONINE (TRI), THYROXINE (TX), AND ANALOGUES IN PREVENTION OF SKELETAL LESIONS IN RATS TREATED WITH 6 MG./DAY OF AMINOACETONITRILE (AAN) FOR 8 DAYS AND 9 MG./DAY AAN FOR FOLLOWING 9 DAYS ADMINISTERED SUBCUTANEOUSLY

Treatment) (g./day/rat)		Average		Severity of lesions
		Starting weight (gram)	Final weight (gram)	
Purina only[1]		61	162	0
Purina + AAN		68	87	4+
Tx 10		68	113	4+
Tx 50		67	133	4+
Tx 100		67	142	1+
TRI 1		67	140	1+
TRI 2		65	106	0
TRI 10		65	90	0
3:5:3′-Triiodothyroacetic acid	10	66	132	1+
	100	66	113	1+
3:5:3′-Triiodothyropropionic acid	10	65	128	4+
	100	65	130	2+
3:5:3′:5′-Tetraiodothyroacetic acid	10	64	112	4+
	100	65	132	1+
3:5:3′:5′-Tetraiodothyropropionic acid	10	63	92	4+
	100	65	135	2+
L-3:3′:5′-Triiodothyronine	10	66	121	4+
	100	66	115	3+
3:3′:5′-Triiodothyropropionic acid	10	65	125	4+
	100	60	136	3+
D,L-3:3′-Diiodothyronine	10	68	120	4+
	100	68	127	3+
3:3′-Diiodothyropropionic acid	10	66	136	4+
	100	63	98	3+

6 rats were used to test each dose. Purina chow was fed *ad lib.*

[1] 0.2 ml. normal saline injected daily.

An attempt was made to use the suppressing effect of thyroid hormones and analogues on the AAN lesions in weanling rats as a "bioassay" for these compounds. For this purpose all the analogues listed in Table 3 were administered also in doses of 2 μg. and

TABLE 4. APPROXIMATE RELATIVE POTENCIES OF THYROXINE AND TRIIODOTHYRONINE ANALOGUES IN THE SUPPRESSION OF AMINOACETONITRILE LESIONS IN RATS

Thyroxine	100
L-3:5:3′-Triiodothyronine	5000
3:5:3′-Triiodothyroacetic acid	100
3:5:3′-Triiodothyropropionic acid	90
3:5:3′:5′-Tetraiodothyroacetic acid	90
3:5:3′:5′-Tetraiodothyropropionic acid	75
L-3:3′:5′-Triiodothyronine	0
3:3′:5′-Triiodothyropropionic acid	0
D,L-3:3′-Diiodothyronine	0
3:3′-Diiodothyropropionic acid	0

50 μg. per day to groups of six rats receiving the AAN treatment. At the end of the experiments an estimate was made of the severity of the skeletal lesions as observed in the roentgenograms and in the histological sections. Although the rows of epiphyseal cartilage cells were distorted in many animals it was possible to make an approximate cell count of several rows and obtain the average number of cells per row. A similar area of the upper tibial epiphyseal plate was counted in each animal. The cell count was checked against the dose of thyroid hormone or analogues received by the animal. Table 4 shows the approximate relative potencies of the compounds tested.

III. *Effect of Corticoids on the AAN Lesions in Rats*

The work of Selye and collaborators (11) on lathyrism stimulated us to test corticosterone and cortisone. Groups of five weanling male Sprague-Dawley rats fed Purina chow were injected daily subcutaneously with 7.5 mg. AAN for the first 18 days of the experiment and 12 mg. AAN for the following 6 days. The daily doses of corticosterone or cortisone indicated in Table 5 were simultaneously injected. Estrone (20 μg. per day) was also tested. Four groups of rats served as controls. One control group received the AAN injections and not the corticoids. The other three control groups did not receive AAN: normal saline (0.2 ml. per day) was injected to the rats of one group, corticosterone

TABLE 5. EFFECT OF INJECTED CORTICOSTERONE, CORTISONE (3) AND ESTRONE IN PREVENTION OF SKELETAL LESIONS IN RATS TREATED WITH 7.5 MG./DAY AMINOACETONITRILE (AAN) FOR 18 DAYS AND 12 MG./DAY FOR FOLLOWING 6 DAYS ADMINISTERED SUBCUTANEOUSLY

Treatment Daily dose/rat		Average		Severity of lesions
		Starting weight (gm.)	Final weight (gm.)	
A) Purina only[1]		64	180	0
Corticosterone[3]	2 mg.	67	195	0
Cortisone[4]	2 mg.	63	97	0
B) Purina + AAN[1]		63	156	4+
Corticosterone	1 mg.	64	178	3+
Corticosterone	2 mg.	67	162	2+
Cortisone[2]	1 mg.	68	129	3+
Cortisone	2 mg.	63	98	2+
Estrone	20 μg.	67	154	4+

5 rats were used to test each dose in the experimental and in the control groups. Duration of experiment, 24 days.

[1] 0.2 ml. normal saline injected daily.

[2] One rat died on the 18th day of the experiment of dissecting aneurysm of the aorta.

[3] Upjohn Company.

[4] Cortone, Merck & Co.

(2 mg. per day) was injected to the rats of another group, and cortisone (2 mg. per day) was injected to the rats of the last group. One rat of the group receiving AAN and 1 mg. per day of the cortisone died of dissecting aneurysm of the aorta on the eighteenth day of the experiment. At the termination of the experiment the animals were sacrificed and roentgenograms were made. The knees were excised and processed for histological studies as in the previous experiments.

The experimental animals which had received corticosterone had skeletal lesions slightly less severe than the animals on AAN alone. The epiphyseal plates of the control rats which had been given corticosterone were somewhat narrower than those of the normal animals. Cortisone markedly slowed the growth of the rats but did not suppress completely the AAN lesions. The epiphyseal plates of the control animals on cortisone were very narrow and the endochondral ossification was greatly deranged (Fig. 3). Estrone was observed to have no suppressing effect on the skeletal lesions produced by AAN.

DISCUSSION

The pathogenesis of the lesions induced in experimental animals by the administration of aminonitriles is not fully understood. The aminonitriles affect a definitely limited stage of the process of mesodermal tissue differentiation, and they do not seem to cause any breakdown of completed mesodermal structures (12). Recent studies indicate that aminonitriles may impair collagen and elastin formation in wound healing (13), in croton oil pouches (14), and in the aorta (12, 15). The epiphyseal plate, being a remnant of continually maturing embryonal tissue, is particularly vulnerable to the action of the aminonitriles (2).

Castellani and Castellani (16) have observed a significant decrease of hexosamine content in epiphyseal plates of young rabbits treated with AAN as compared with the normal; this suggests a decrease in the content of mucopolysaccharides. The same authors have found the hydroxyproline (hence total collagen) content of epiphyseal plates from rabbits treated with AAN not reduced from the normal. This observation has been corroborated in rats by Follis and Tousimis (17) who at the same time have detected with the electron microscope a great reduction of collagen fibrils in the epiphyseal cartilage homogenates of AAN-treated rats. It is suggested that the aminonitriles may cause a failure of the tropocollagen molecule to form collagen fibers. Perhaps the defective mucopolysaccharides of the epiphyseal cartilage are responsible for this failure.

TRI had by far the greatest suppressing effect upon the AAN lesions of all the hormones tested. Tx in doses 50 times greater had a similar effect. However, TRI and Tx caused only a slight suppression of the lesions caused by BAPN. This suggests that there may be a difference in the nature of the biochemical derangement caused by these two aminonitriles although histologically the lesions appear similar. Thus a new approach be-

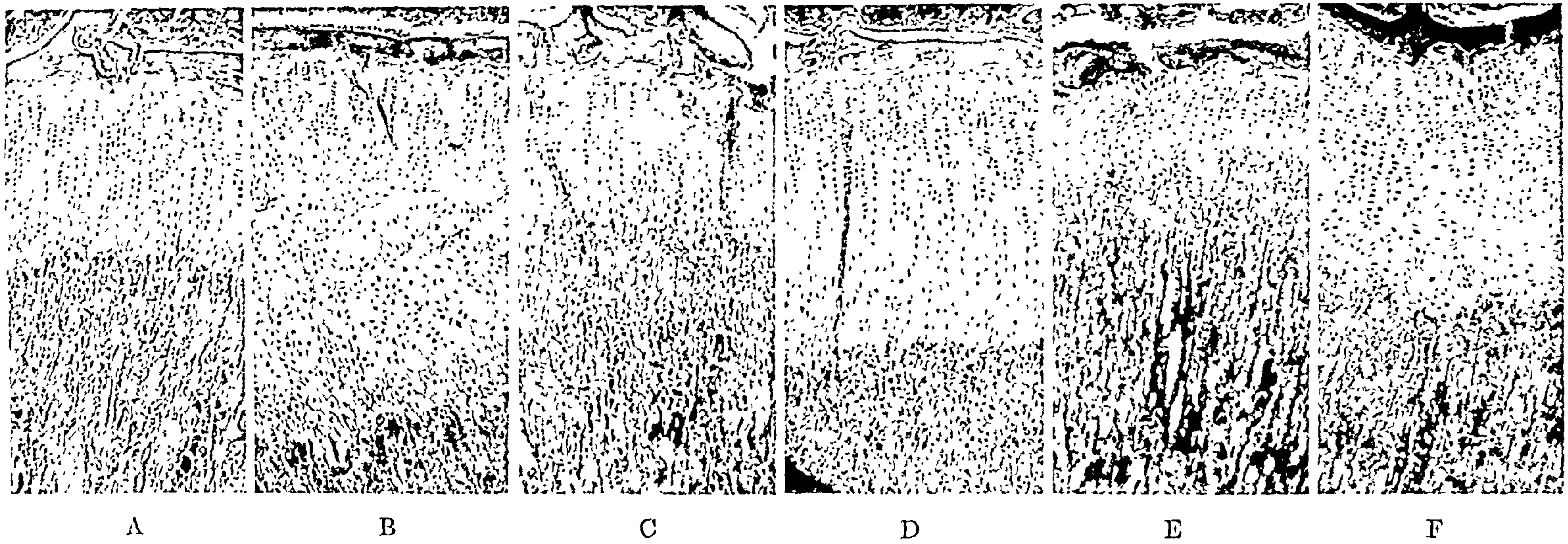

FIG. 3. Proximal epiphyseal plates of the tibia of 55-day-old rats (×30, hematoxylin and eosin stain) treated as follows for 24 days:
A. Normal control.
B. AAN (7.5 mg. per day) for 18 days and AAN (12 mg. per day) for the following 6 days administered subcutaneously.
C. Corticosterone (2 mg. per day) administered subcutaneously.
D. AAN as in B plus corticosterone (2 mg. per day) administered subcutaneously.
E. Cortisone (2 mg. per day) administered subcutaneously.
F. AAN as in B plus cortisone (2 mg. per day) administered subcutaneously.

comes available for study of the action of thyroid hormones upon connective tissues. The suppression of the AAN lesions by Tx and TRI may be related to the effects of these hormones in promoting maturation (18, 19). No explanation can be offered at this time why there is only a very slight suppressing effect of these hormones on the lesions produced by BAPN.

The activity of Tx and TRI analogues tested in our experiments corresponded closely with the activity of these compounds in the suppression of the thiouracil-induced goiter in rats (10). Roche *et al.* (10) reported that 3:5:3′-triiodothyropropionic acid was 290 times more active than Tx in its effect on the metamorphosis of tadpoles. The activity of these two compounds was found to be almost equal in the suppression of the AAN lesions in rats.

Selye and Bois (11) have described the suppression with cortisol acetate of the skeletal lesions produced by AAN in rats with one kidney removed and given a 1% NaCl solution as drinking fluid. In our experiments with intact rats neither corticosterone nor cortisone suppressed the AAN lesions completely, although these were less severe than in control animals receiving AAN alone. Inhibition of bone growth and structural changes in the metaphysis produced by cortisone have been previously described (20, 21, 22). We observed great narrowing of the epiphyseal plates and the derangement of the endochondral ossification in the control rats injected with cortisone. However, the AAN-cortisone treated rats had epiphyseal plates wider than the normal animals, with disarranged cartilage cell columns. Corticosterone was found to be less effective than cortisone in the control of the epiphyseal plate lesions. Testosterone failed in previous experiments (2) to arrest the lesions produced in rats with *Lathyrus odoratus* meal. Estrone was found likewise to be ineffective in checking the lesions produced by AAN in the experiments here reported.

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THE EFFECT OF ADRENAL AND PITUITARY HORMONES ON THE URINARY EXCRETION OF IODIDE IN MICE[1]

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ABSTRACT

The urinary excretion of radioiodide was measured in mice injected with a variety of adrenal and pituitary hormones.

While radioiodine excretion was diminished in adrenalectomized mice and restored to a nearly normal rate by cortisone, this hormone, as well as aldosterone, deoxycorticosterone, adrenaline and noradrenaline, had little effect in intact mice. ACTH was slightly ioduretic and TSH had no effect.

A considerable increase in radioiodide excretion was observed after acute injection of the posterior pituitary hormones, vasopressin and oxytocin. The effect of vasopressin did not depend on the amount of impurity present in the preparations tested, and varied linearly with the logarithm of the dose. This hormone increased the renal clearance of radioiodide from blood. These observations suggest that the posterior pituitary plays a role in the control of iodide excretion.

When vasopressin was administered for prolonged periods of time, the resulting iodide losses, unlike those produced by sodium chloride administration, did not induce a stimulation of the thyroid gland. In fact, vasopressin depressed the thyroid gland since the thyroid weight was reduced to about 40% of that in controls. Presumably, this effect prevented the iodine losses from resulting in thyroid stimulation.

THE urinary excretion of iodide in mice is increased 10 to 20 times by a single dose of sodium chloride (1). When this salt is administered over a long period of time, the iodide losses induce a relative iodine deficiency which may result in thyroid stimulation and even goiter (1, 2).

This work was followed up by investigating the influence of various factors on ioduria. When a number of salts other than sodium chloride were tested, the results obtained led to the conclusion that ioduresis is induced by an increase in the ionic concentration of blood, no matter which ions produce this increase (3). The effect of hormonal factors on ioduria was

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The assistance of Mr. Roy Blake at some phases of this investigation as well as the supply of vasopressin by Dr. B. G. Benfey, of noradrenaline by Dr. A. S. V. Burgen, of aldosterone by Dr. I. Dyrenfurth and of pitressin and pitressin tannate by Parke, Davis and Co. is gratefully acknowledged.

examined in the experiments now to be reported. The posterior pituitary hormone vasopressin was found to increase the renal excretion of iodide considerably. It was therefore investigated whether or not prolonged treatment with this hormone can result in thyroid stimulation and goiter, as in the case of treatment with sodium chloride.

MATERIAL AND METHOD

The hormones were tested in 2 to 9-month old C_3H and albino mice. Males were used in all experiments except one (ascorbic acid, Table 1). Since sodium chloride was known to increase the urinary excretion of iodine (1), the mice were fed for 3 weeks prior to the hormone administration on a diet containing only traces of sodium chloride (2). This diet, which contained less than 3 µg. iodine per 100 gm., consisted of 89% corn meal, 10% dried brewer's yeast and 1% calcium carbonate. However, two of the experiments (reported in Fig. 1 and Table 3) were carried out in mice fed Purina fox chow.

In all acute experiments, each mouse received simultaneously 2 subcutaneous injections of 0.2 ml. aqueous solutions containing respectively the hormone and 10 µc. carrier free I^{131}. The animal was then placed in a 250 ml. beaker fitted with a wire mesh floor allowing the urine to settle at the bottom and was given access to distilled water but received no food. Two and a half hours later, the mouse was anaesthetized with ether in the beaker, and the thyroid gland was removed. The I^{131} content of urine (including that in the bladder) and of the thyroid was then measured as described previously (1).

The effect of sodium chloride on the renal clearance of radioiodine from the blood was investigated by measuring the radioiodine content of urine and blood 1 and 3 hours after I^{131} administration as described earlier (1).

The following hormones were used: *vasopressin:* Pitressin from Parke, Davis (55 U.S.P. units per mg.); in one experiment (Table 3) two preparations (200 and 400 U.S.P. units per mg.) obtained from Dr. B. G. Benfey, Department of Pharmacology, McGill University, were also tested. *Vasopressin tannate* in peanut oil: Parke, Davis. *Oxytocin:* Dr. B. G. Benfey. *Thyrotrophic hormone* (TSH): Nordic Biochemicals, Montreal. *Adrenocorticotrophic hormone* (ACTH): Armour. *Aldosterone:* Dr. I. Dyrenfurth, Department of Investigative Medicine, McGill University. *Deoxycorticosterone acetate:* Nutritional Biochemicals Corp. *L-adrenaline:* Eastman. *D,L-noradrenaline:* Dr. A. S. V. Burgen, Department of Physiology, McGill University. *Cortisone acetate:* Merck.

RESULTS

Adrenal hormones (Table 1)

Urinary excretion and thyroid uptake of radioiodine were not significantly influenced by cortisone, but were significantly decreased by adrenalectomy (performed immediately before treatment) while the adminstration of cortisone to adrenalectomized animals restored a normal condition.

The other adrenal hormones were tested in intact animals. They did not influence urinary excretion, except for adrenaline which decreased it.

The thyroid uptake of radioiodine was decreased by adrenaline and noradrenaline[2] and slightly increased by aldosterone. No effect of ascorbic acid was observed.

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[2] The effect of adrenaline and noradrenaline was reported in detail elsewhere (4).

Pituitary hormones

Adrenocorticotrophic hormone and two posterior pituitary hormones, vasopressin and oxytocin, significantly increased the urinary excretion of radioiodine, while thyrotrophic hormone had no effect (Table 2). None of these hormones modified the thyroid uptake of radioiodine in the experiment reported in Table 2, but in other experiments vasopressin significantly decreased it (4).

TABLE 1. THE ACUTE EFFECT OF ADRENAL HORMONES ON THE URINARY EXCRETION AND THYROID UPTAKE OF RADIOIODINE

Treatment	No. of mice*	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
Sham adrenalectomy, Controls	6	10.1 ±2.8	11.9 ±1.9
Sham adrenalectomy, 2 mg. cortisone acetate	6	7.8 ±1.8	11.6 ±1.5
Adrenalectomy†	6	*3.2 ±1.0*	*7.0 ±1.1*
Adrenalectomy†, 2 mg. cortisone acetate	6	7.0 ±2.4	13.3 ±2.5
Controls	8	2.4 ±0.6	11.7 ±2.0
1 mg. deoxycorticosterone acetate‡	7	1.1 ±0.4	15.2 ±2.4
Controls	8	5.1 ±0.7	19.7 ±2.1
1 μg. aldosterone	8	3.3 ±0.6	*27.6 ±2.2*
Controls	8	4.0 ±0.5	20.5 ±1.2
1 μg. adrenaline	8	2.6 ±0.5	*16.3 ±1.2*
50 μg. adrenaline	8	*1.4 ±0.2*	*5.7 ±0.7*
0.9 μg. noradrenaline	8	4.7 ±1.0	16.8 ±1.8
46 μg. noradrenaline	8	4.6 ±0.8	*11.6 ±1.1*
Controls	8	3.1 ±0.6	10.4 ±3.6
1 mg. ascorbic acid	8	2.6 ±0.4	10.6 ±5.8

* C_3H mice aged 4, 9, 9, 5 and 9 months respectively from the first to the fifth experiment.
† Adrenals removed immediately prior to treatment.
‡ Dissolved in polyethyleneglycol. Controls received polyethyleneglycol.
The italicized figures are significantly different from those of the respective controls.

The effect of vasopressin was further investigated by injecting 1 U.S.P. unit of 3 different preparations of the hormone. In spite of differences in the purity of the three preparations, they increased the urinary excretion of radioiodine to the same extent (Table 3). Thus it was concluded that the active principle was not an impurity but pitressin itself.

When graded doses of vasopressin were injected to Purina fed mice, the amount of radioiodine excreted in the urine varied linearly with the logarithm of the dose (Fig. 1), the increment being 9% of the amount of radioiodine injected for a 10-fold increase in vasopressin dose.

The effect of vasopressin on the renal clearance of radioiodine from the blood was investigated by measuring the urine content and the blood level of this isotope at 1 and 3 hours following administration of the hormone. During the two-hour period comprised between the first and third hour,

TABLE 2. THE ACUTE EFFECT OF PITUITARY HORMONES ON THE URINARY EXCRETION AND THYROID UPTAKE OF RADIOIODINE

Treatment	Number of mice*	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
Controls	8	5.1 ±0.7	19.7 ±2.1
1 U.S.P. unit TSH	8	7.3 ±0.9	15.1 ±2.7
1 mg. ACTH	7	*11.7 ±2.7*	22.9 ±4.1
1 U.S.P. unit vasopressin	7	*20.2 ±3.1*	16.4 ±1.5
1 U.S.P. unit oxytocin	8	*16.5 ±2.1*	23.0 ±1.4

* C_3H 9-month-old mice.
The italicized figures (urine I^{131}) are significantly different from that of controls.

the mean volume of blood cleared per minute by the kidney was 7 microliters in controls and 27 microliters in vasopressin treated animals (Table 4). Thus, the hormone produced a 3.7-fold increase in the renal clearance of radioiodine.

Prolonged administration of vasopressin

Since vasopressin considerably raised the excretion of iodide and since it had been shown previously (2) that large and prolonged losses of iodide caused by sodium chloride ingestion may lead to thyroid stimulation and goiter production, there was a possibility that prolonged administration of vasopressin would also induce goitrous thyroids. This possibility was investigated in mice fed the diet described above, and injected subcutaneously with 0.5 unit vasopressin in 0.05 ml. water twice daily for 50 days. Controls were injected 0.05 ml. water at the same rate. After 50 days all animals were given a subcutaneous injection of 10 μc. radioiodine and sacrificed 2.5 hours thereafter. The left thyroid lobe was dissected free of connective tissue, weighed on an analytical balance and homogenized in sodium hydroxide solution for radioactivity counting. The average weight per thyroid lobe was 1.7 ±0.8 in controls and 1.1 ±0.2 in vasopressin treated mice. The respective thyroid uptakes of radioiodine in the two groups were 33.3 ±1.8 and 23.5 ±5.3. Thus, vasopressin did not stimulate the thyroid gland.

TABLE 3. THE ACUTE EFFECT OF 1 U.S.P. UNIT OF VARIOUS VASOPRESSIN PREPARATIONS ON THE URINARY EXCRETION OF RADIOIODINE

Vasopressin preparation	Number of mice*	% of injected radioiodine in	
		Urine Mean ±S.E.	Thyroid Mean ±S.E.
Controls	8	17.1 ±5.7	1.4 ±0.4
Parke Davis (55 U.S.P. units/mg.)	8	*41.0 ±4.1*	2.4 ±0.5
Benfey (200 U.S.P. units/mg.)	8	*43.4 ±4.3*	1.5 ±0.4
Benfey (400 U.S.P. units/mg.)	8	*42.3 ±4.6*	1.2 ±0.3

* C_3H, 5-month-old, Purina fed mice.
The italicized values (urine I^{131}) are significantly different from that of controls.

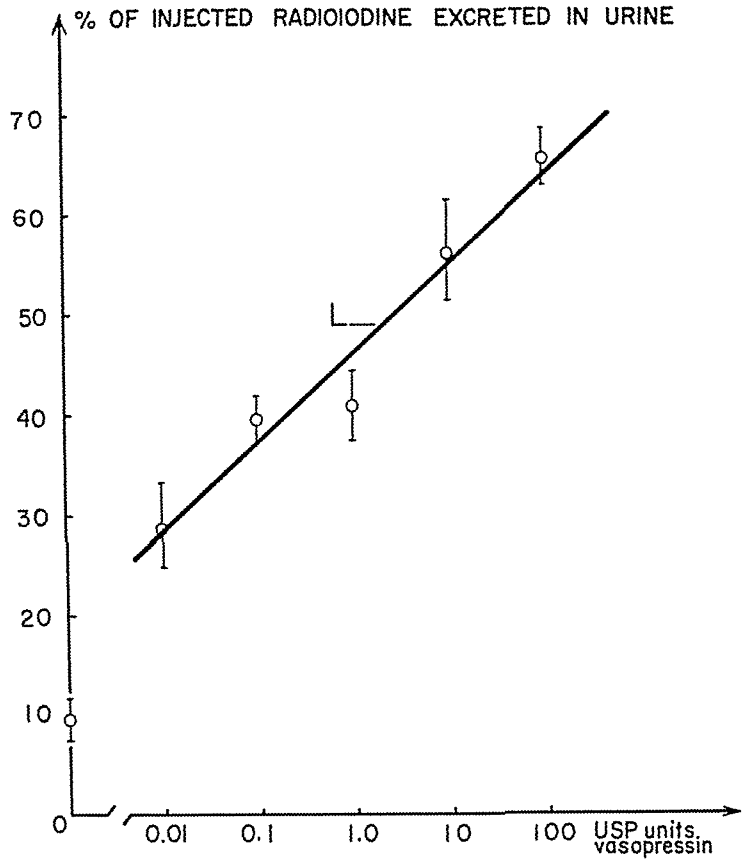

FIG. 1. The acute effect of graded doses of vasopressin on the urinary excretion of radioiodine. The dots indicate the average I^{131} excretion obtained in 7 to 8 albino, 3-month-old, Purina fed mice and the vertical lines the standard errors of the mean. The line L is the best fit line according to the criterion of least squares obtained with the graphic method of Askovitz (14).

The lack of thyroid stimulation after chronic vasopressin administration might be due to the fact that vasopressin eventually became ineffective in augmenting the urinary loss of iodide. However, after one month treat-

TABLE 4. ACUTE EFFECT OF VASOPRESSIN ON BLOOD RADIOIODIDE CLEARANCE BY THE KIDNEY

Treatment	Sacrifice time (hours)	Number of mice*	% of injected I^{131} in		Blood I^{131} renal clearance (ml./minute)
			Urine Mean ±S.E.	1 ml. blood Mean ±S.E.	
Controls	1	7	7.9 ±2.1	3.8 ±0.4	0.007
	3	7	11.2 ±2.8	3.7±0.5	
1 U.S.P. unit vasopressin	1	8	27.9 ±2.3	2.8 ±0.3	0.027
	3	8	35.9 ±4.3	2.3 ±0.5	

* Albino, 2–3-month-old.

TABLE 5. THE ACUTE EFFECT OF VASOPRESSIN ON THE URINARY EXCRETION OF RADIOIODINE IN MICE PREVIOUSLY ADMINISTERED 1 U.S.P. UNIT VASOPRESSIN DAILY FOR 1 MONTH*

Acute treatment	Number of mice†	% of injected radioiodine in urine Mean ±S.E.
Controls	4	10.7 ±0.6
1 U.S.P. unit vasopressin	4	47.9 ±4.1

* Chronic vasopressin treatment was discontinued during the two days preceding acute vasopressin injection.
† Albino, 2–3-month-old.
Vasopressin significantly increased the urinary excretion of radioiodine.

ment, an acute administration of vasopressin to radioiodine injected mice still elicited the usual ioduresis (Table 5). This hypothesis was therefore rejected.

Another possible explanation for the lack of thyroid stimulation might be the rapid disappearance of vasopressin from the body after injection. A chronic experiment was then carried out as above except for the use of a hormone preparation effective over relatively long periods of time, namely, vasopressin tannate in oil. The effective period of time was determined in a preliminary experiment by injecting 1 U.S.P. unit vasopressin tannate in 0.2 ml. oil and then allowing various periods of time before injecting radioiodine and sacrificing 2.5 hours later. When allowing 4 hours between vasopressin tannate and radioiodine injection, a significant increase in urinary radioiodine was observed. That is the hormone was still effective between 4 and 6.5 hours following injection (Fig. 2). After 8 hours a slight and after 24 hours a very slight effect was observed but these were not significant statistically. After 48 hours definitely no effect was noticeable (Fig. 2). Accordingly, it was decided to administer the preparation once a day. One U.S.P. unit vasopressin tannate in 0.2 ml. oil was injected subcutaneously daily for 30 days to 12 mice. Twelve control mice received a daily dose of 0.2 ml. peanut oil.

TABLE 6. THE EFFECT OF 30 DAYS DAILY ADMINISTRATION OF VASOPRESSIN TANNATE IN OIL ON THE THYROID GLAND

Treatment	Number of mice*		Body Weight (gm.)		Left thyroid lobe weight (mg.) Mean ±S.E.	% of injected radioiodine in thyroid† Mean ±S.E.
	Initial	Final	Initial Mean ±S.E.	Final Mean ±S.E.		
Controls (0.2 ml. oil daily)	12	11	24.6 ±1.0	28.0 ±1.1	0.98 ±0.11	12.2 ±1.8
1 U.S.P. unit vasopressin tanate in 0.2 ml. oil daily	12	5	25.6 ±0.5	*21.2 ±1.1*	*0.38 ±0.06*	9.3 ±0.7

* Albino, 2 months old at the start.
† Left lobe only.

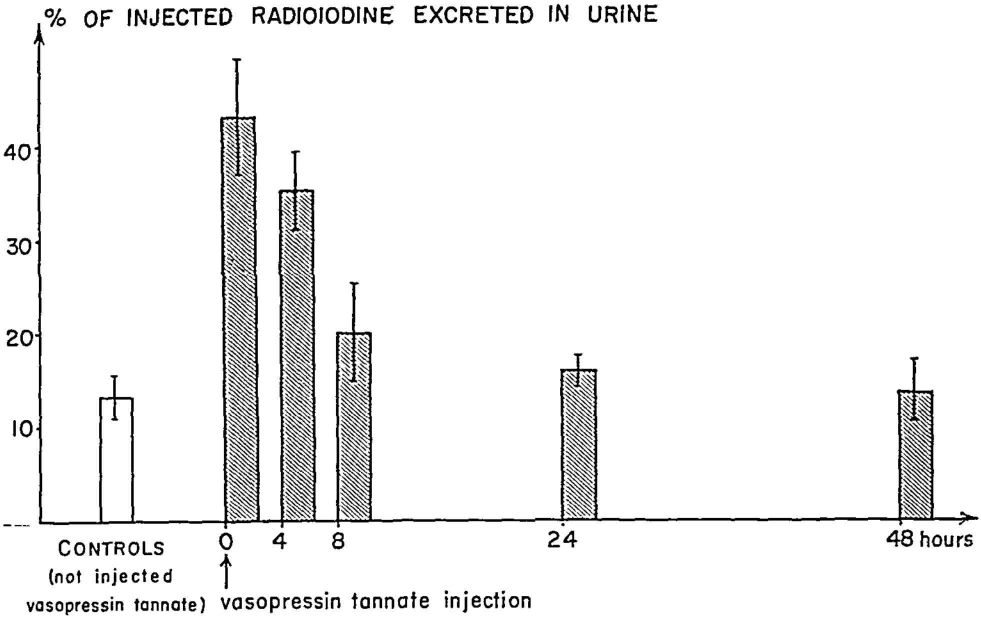

FIG. 2. Duration of effect of 1 U.S.P. unit vasopressin tannate in oil on the urinary excretion of radioiodine. The height of the bars indicates the average I^{131} excretion observed in 7 to 8 albino, 4-month-old mice. The vertical lines are the standard errors. Vasopressin tannate was injected at time 0. (The left side of the bars indicates the time of I^{131} injection and the right side the time of sacrifice, that is, 2.5 hours later.)

While the control mice gained weight and only 1 out of 12 died, the vasopressin-tannate-treated mice lost weight and 7 out of 12 died over the 30-day period. The thyroid weight was significantly lower in vasopressin-tannate-treated mice than in controls. Thyroid uptake of radioiodine was slightly decreased (Table 6). Incidentally, during the course of the experiment, it was noted that the hairs of the vasopressin treated mice were covered with oil, while those of controls were dry. At autopsy large amounts of oil were found subcutaneously in controls, but not in vasopressin treated animals. Perhaps the hormone modified the skin permeability to oil or prevented closure of the holes made by the injection needle.

DISCUSSION

Although adrenal hormones are known to influence the excretion of sodium and other electrolytes (5–8), they had little effect on the excretion of the iodide ion in the intact mouse. The observation that iodide excretion is decreased by adrenalectomy and is restored by cortisone to nearly the same level as in intact mice is in agreement with a similar observation made by Paris *et al.* (9) in the rat and with the fact that cortisone enhances radioiodine excretion in hypophysectomized rats (10). Thus it appears that the presence of intact adrenals is required for an adequate excretion of the iodide ion. However, exogenous adrenal hormones have little effect in animals with adrenals functioning adequately: cortisone, deoxycorticosterone acetate and aldosterone did not modify iodide excretion. (The moderate decrease in excretion produced by adrenaline is likely to be the result of vasoconstriction.)

In contrast, posterior pituitary hormones considerably enhanced the excretion of iodide in intact animals. The mechanism by which vasopressin produces this effect cannot be explained merely on the basis of alterations in the concentration of plasma radioiodide resulting from changes in the extrarenal metabolism of iodide (e.g. in inter-/intracellular distribution (11)) but involves a change in functional activity of the kidney, since the clearance of radioiodide was increased. The fact that posterior pituitary hormone also increases the excretion of chloride (12) as well as sodium and potassium (13) suggests that these hormones may influence the urinary excretion of electrolytes in general.

The role of the anterior pituitary on iodide excretion is not clear. While ACTH increased iodide excretion (Table 2) and growth hormone was found by others to have the same effect in hypophysectomized rats (9), it was not demonstrated that the preparations used were pure, and their effect might be due to contamination by posterior pituitary hormones.

Chronic effect of vasopressin

It was possible that, as in the case of mice given sodium chloride in the diet (1, 2), the mice treated for prolonged periods of time with vasopressin might suffer such iodide losses that a relative iodine deficiency would occur which might result in thyroid stimulation. Such a stimulation did not occur. On the contrary, the decrease in the weight and radioiodine uptake of the thyroid gland observed after prolonged treatment with vasopressin indicates that the thyroid activity was depressed. The high mortality (58%) and the loss of weight in the animals administered the hormone indicate that this treatment resulted in general damage or stress, which may account for the thyroid depression. Presumably, this effect prevented the gland from being stimulated by the iodine losses.

In conclusion, the urinary excretion of the iodide ion was significantly increased by the posterior pituitary hormones vasopressin and oxytocin, while it was but little influenced by adrenal hormones.

Acknowledgment

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HEPATIC REGULATION OF ADRENAL CORTICAL FUNCTION[1,2]

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ABSTRACT

Under many different experimental and physiological conditions, a close correlation has been found between the size of the adrenal glands and the capacity of the liver to inactivate adrenal cortical hormones by *ring A reduction in vitro*. We have concluded from this close correlation that there is a similar parallel relationship between the *in vivo* capacity of the liver to inactivate corticosteroids, and the adrenal cortical secretion rate. This conclusion is based on several assumptions which are discussed. The present experiments demonstrate that this relationship denotes secondary responses of the adrenal cortex to primary, independent variations in hepatic inactivation of corticosteroids. Neither ACTH nor adrenal cortical hormones increases the capacity of the liver to inactivate corticosteroids. In contrast, however, a primary diminution of hepatic capacity for adrenal steroid inactivation by surgical removal of 65% of the liver tissue can cause secondary adrenal atrophy. Therefore, the parallel relationship between hepatic capacity for inactivation of adrenal cortical hormones by ring A reduction, and the size of the adrenal glands represents a controlling influence of the liver on adrenal cortical function, rather than the reverse.

This hepatic regulation of adrenal cortical function is apparently mediated through the anterior pituitary (ACTH) negative-feedback control which stabilizes plasma corticosteroid concentration. Our data indicate that the control very effectively matches the mean input of adrenal cortical hormones to any given output over wide ranges of steroid inactivation rates. By means of these data, the automatic control properties of the hepatic-anterior pituitary-adrenal cortical system are analyzed to show that the amount of TPNH-bound Δ^4-steroid hydrogenases in the liver may affect: a) the time constant of the system (the time required to diminish by 63% a deviation from the mean plasma corticosteroid concentration); b) the extent of hunting of plasma corticosteroid concentration about a mean value; c) the half-life of the steroid molecules in the system; d) the velocity of their inactivation at any given plasma corticosteroid concentration; and, e) the mean concentration of adrenal cortical hormones in the blood.

IT HAS been proposed that the concentration of the adrenal cortical hormones in blood is stabilized by a negative-feedback control of adrenal cortical secretion rate, so that changes in the rate of secretion are brought about by opposite changes in the corticosteroid concentration in blood (2,

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3). The concentration of these steroids in blood depends upon both the rate of secretion of the hormones into blood, and the rate of removal of them from blood. Since urinary and biliary losses of active corticosteroids are negligible (4, 5), the removal of adrenal steroids is accomplished almost entirely by the enzymatic processes which inactivate the molecules. The main site of corticosteroid inactivation is the liver (6, 7). Therefore, if it is true that the rate of adrenal cortical secretion is regulated by the proposed negative-feedback control, changes in the rate of hepatic metabolism of these hormones should cause parallel changes in the rate of adrenal cortical secretion. The demonstration of this predicted relationship would establish the liver as a principal determinant of adrenocorticotrophic hormone (ACTH) release and adrenal cortical secretion rate in normal animals, and provide new support for the negative-feedback control hypothesis.

Studies of patients with liver or thyroid disease (4, 7, 8) have suggested that the liver exerts some influence on adrenal cortical function. Under a wide variety of physiological and experimental conditions, we have found a remarkably close parallel relationship between the capacity of the liver to inactivate adrenal cortical hormones *in vitro* by ring A reduction, and the size of the adrenal glands (9, 10). Female rats have larger adrenals than males, and their livers have a greater *in vitro* capacity to inactivate corticosteroids (10). In hamsters these hepatic and adrenal sex differences are both reversed: male hamsters have larger adrenals and greater capacity to inactivate corticosteroids than have females (10). Hyperthyroid rats of each sex have both larger adrenals and greater capacity for steroid metabolism by liver than do normal rats of the same sex. In hypothyroid animals both adrenal size and steroid metabolism by liver are diminished. The correlation between adrenal size and the capacity of the liver to inactivate the corticosteroid substrate was extraordinarily close in those experiments ($r = 0.97$) (9).

In addition to the above evidence, we have also found that castration of young rats abolishes both the hepatic and adrenal sex differences. These sex differences can then be re-established together by treating the castrated males with androgens, and the castrated females with estrogens, or be completely reversed together by giving the estrogen to the castrated males, and the androgen to the spayed females (10). Furthermore, in both sexes of caesarian-delivered rats raised on a pasteurized chow diet, the livers comprise a larger fraction of total body weight, and have higher Δ^4-steroid hydrogenase activity than is usual for the strain after normal birth and growth on an ordinary chow diet. The relative adrenal size of such animals is also larger than is usual for the strain (unpublished data). Finally, even the rapid adrenal enlargement of the female rat at estrus is associated with a parallel increase in total hepatic Δ^4-steroid hydrogenase activity, as shown in the experiments to be described.

From this very clear relationship between the *in vitro* capacity of the liver for the reduction of adrenal cortical steroids at ring A, and the size of the adrenal glands, we have concluded that there is a similarly close relationship between the *in vivo* capacity of the liver to inactivate adrenal cortical hormones, and the secretion rate of the adrenal cortex. This conclusion is based on three assumptions, in addition to the evidence presented above. The assumptions are: a) ring A reduction is a major reaction leading to inactivation of corticosteroids in liver; b) changes in total adrenal weight are an indication of parallel changes in adrenal cortical secretion rate in the conditions and species studied; and c) judgments about hepatic metabolism of adrenal steroids *in vivo* can be made from these *in vitro* assays. Insofar as these assumptions are true,[3] a close parallel relationship between hepatic capacity for inactivation of corticosteroids and adrenal cortical secretion rate appears to be established.

Before this close relationship can be taken as evidence for the hepatic regulation of adrenal cortical function, it must be shown that primary changes in hepatic capacity for corticosteroid inactivation can cause parallel changes in adrenal cortical secretion rate, but that primary alterations of plasma corticosteroid concentration do not cause parallel changes in hepatic inactivation of these hormones. The present experiments were undertaken to test these two propositions, and through them, the prediction that the liver regulates adrenal cortical function.

METHODS

Adult male and female Sprague-Dawley rats weighing 200–500 gm. were used in all experiments. The animals were kept in a thermoregulated room (22° C) and were maintained on Purina chow and tap water.

All assays of hepatic capacity for the *in vitro* inactivation of adrenal cortical hormones were performed in duplicate. Corticosterone was the substrate, liver slices were the source of enzymatic activity, and the loss of spectral absorption of the steroid at 240 mμ was the measure of ring A reduction. Animals were killed by a blow on the head and their livers were quickly excised, rinsed in iced saline, and weighed. Slices were cut on a Stadie-Riggs hand microtome, and 200 ± 5 mg. of slices were added to each incubation flask containing 2 ml. of warmed, oxygenated phosphosaline buffer (19). Each 2 ml. of the buffer contained 18.2 μM glucose, 0.368 μM corticosterone, and a trace (0.4% v/v) of ethanol.

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[3] The products of ring A reduction are inactive at physiological concentrations (11), and the reaction is for practical purposes irreversible (12). Although reduction of the C_{20} carbonyl group may occur without prior reduction of ring A (13, 14) and is sufficient to inactivate corticosteroids (15), ring A reduction appears to be the first alteration of the steroid molecules in the major *in vivo* pathway for metabolism of adrenal steroids (16). Subsequent reductions of the 3 and 20 ketones, and the conjugation with uridine diphosphoglucuronic acid which occurs in most of the species studied, serve to increase the water solubility of the inactivated molecules and facilitate excretion of them from the body.

Under some experimental conditions, changes in adrenal size do not give an accurate indication of changes in adrenal cortical secretion rate (17). However, adrenal cortical secretion is controlled by ACTH, and from the data of Sayers *et al.* (18) it can be shown that adrenal size is linearly related to the log of the dose of ACTH in hypophysectomized rats. If time for the change in gland size is allowed, the assumption that changes in gland size indicate sustained changes in secretion rate, under most physiological conditions, is reasonable.

The reaction mixtures were incubated for 15 minutes at 37.5° C under 100% oxygen at ambient pressure, with continuous shaking. Similar mixtures were held at 0° C for 15 minutes. The reactions were stopped and the extraction of unmetabolized steroid was begun by the addition of 20 ml. of cold redistilled dichloromethane to each vessel. Thereafter, the procedures were exactly as described previously for experiments with liver homogenates (20). Recovery of known amounts of added steroid from the 0° C mixture was 98% complete. The background readings contributed 13% of the total optical density of the 0° C mixture at 240 mμ.

The reaction velocity in these assays is very dependent upon the initial substrate concentration, and it was necessary to reproduce this concentration very accurately in every experiment. In spite of this difficulty we have found that the slice assays are preferable to homogenate assays, in which zero-order kinetics can be achieved (20), for the purposes of these experiments. The reaction velocity in the slices is affected by changes in the availability of both the enzyme and the coenzyme, TPNH (reduced triphosphopyridine nucleotide) (9), involved in the reaction. The homogenate assay can accurately measure only variations in the amount of the enzyme. We have previously shown that the amounts of enzyme and available coenzyme may be changed independently under various experimental conditions (9). For this reason the slice assay gives a better *in vitro* estimate of the *in vivo* effect of a treatment than does the homogenate assay.

The expression of total hepatic capacity for the *in vitro* reduction of corticosterone at ring A is obtained by multiplying the amount of metabolism in 200 mg. of slices in 15 minutes (the activity concentration) by

$$\frac{(5)(\text{total liver weight in gm.})(100)}{(15)(\text{body weight in gm.})}.$$

The final activity unit is therefore: μM corticosterone reduced at ring A per minute per 100 gm. body weight. This form of expression for the total hepatic capacity for steroid ring A reduction is independent of any quantitative change in liver tissue constituents, such as glycogen, which may have occurred during an experiment. An increase in liver glycogen, for example, would increase liver size, but the accompanying decrease in activity concentration would leave the calculation of total capacity unaffected.

For interpretation of the results it is necessary to distinguish between the hepatic capacity for corticosteroid inactivation, as calculated above, and the instantaneous rate of hepatic inactivation of the hormones. The hepatic capacity is a measure of the hepatic content of active enzyme (enzyme plus necessary cofactors) involved in the metabolism of the steroid molecules. The instantaneous reaction rate *in vivo* depends both upon this capacity, and upon the existing substrate concentration in the blood, since the removal of corticosteroids from blood is a first-order process (21, 22). An increase in blood concentration of adrenal cortical hormones would be followed by an increased rate of removal of them from blood, even at constant hepatic capacity for adrenal steroid inactivation. The hepatic capacity, however, determines the reaction rate for any given substrate concentration, and also determines the half-life of the hormones at all concentrations.

Animals treated with ACTH received 10 U./day of Organon ACTH dissolved in

1 ml. of gelatin diluent (16% gelatin, 30% propylene glycol, 0.5% phenol) subcutaneously. Animals treated with cortisone acetate received either 1.5 or 3.0 mg./day of the Upjohn microcrystalline suspension subcutaneously. Adrenal enucleation and contralateral adrenalectomy were performed at one stage through bilateral dorsal incisions, under ether anesthesia. The blood clot and necrotic center were removed from the regenerating adrenals at the time of weighing, 6 or 12 days after operation. It was often difficult to separate cortical tissue from debris, and the weights recorded are subject to some uncertainty. Partial hepatectomy with removal of approximately 65% of the liver tissue was accomplished by the technique of Higgins and Anderson (23).

Rats receiving the acute, 4-hour intravenous infusions of corticosterone were prepared 18 hours before the start of the experiment by catheterization of the lateral caudal vein with a polyvinyl catheter, under ether anesthesia. The animals were then placed in comfortable restraining cages in which they had previously been trained to remain quiet while without food or water. At the start of the experiment, the catheters were unsealed without disturbing the rats, and connected to a constant infusion pump delivering approximately 2 μg. corticosterone per minute at a flow rate of 0.05 ml./minute of 0.15 *M* saline. This infusion rate was chosen to approximate the maximum rate of secretion of corticosterone by both adrenals in rats of the size employed (24, 25). Control animals received infusions of saline only.

Data presented as means are accompanied by the standard errors of the means.

RESULTS

Control groups: effects of age, sex, and estrus

The liver and adrenals represented larger fractions of body weight in young adult males than in older adult males. The activity concentration was essentially the same in both groups (Table 1), so the total hepatic capacity for steroid metabolism and the relative adrenal weight (total adrenal weight in mg./100 gm. body weight) both appeared to decrease slightly with age. This effect of aging was small and could be ignored as long as all experimental animals weighed between 200 and 500 gm.

Female rats had larger adrenals and greater total hepatic capacity for corticosterone ring A reduction than did males (Figs. 1, 2). During estrus, both the adrenal size and total hepatic activity became still greater. At estrus, the adrenal weight increased by 20%, and the total hepatic capacity for steroid inactivation increased by 23% over the levels at diestrus, through an increase in liver size at constant activity concentration (Table 1; Fig. 2, controls). Since the proportion of females in estrus to those in diestrus is small in a random population of rats, no attempt was made to select animals for the experimental groups according to their stage in the cycle. Results obtained in experimental groups of females are compared to the diestrus control group.

Hyperadrenalcortical groups

Prolonged hyperadrenalcortical states were produced in each sex in two different ways. One group of animals was treated for 12 days with ACTH. These animals showed the expected adrenal hypertrophy, and presumably

TABLE 1. RAW DATA SHOWING EFFECTS OF VARIATIONS IN ADRENAL CORTICAL STATE IN BOTH SEXES

Group		No. of rats	Duration (days)	Body wt. (gm.)		Liver weight (gm.)	Total adrenal weight (mg.)	Activity *concentration* (μM corticosterone reduced in 15 min. by 200 mg. of slices)
				Initial	Final			
Males								
Controls	Young adult	5		204 ± 5		9.5 ± 0.4	34.0 ± 2.1	0.12 ± 0.01
	Adult	5		478 ± 24		17.9 ± 1.0	63.3 ± 4.8	0.14 ± 0.01
	Sham-op.	4	6	357 ± 7		12.8 ± 0.4	47.4 ± 6.5	0.17 ± 0.003
Hyper Adrenal Cortical	ACTH 10U./day	5	12	425 ± 8	425 ± 28	16.0 ± 0.2	89.5 ± 4.6	0.10 ± 0.01
	Cortisone Acetate 1.5 mg./day	5	12	311 ± 2	350 ± 10	14.2 ± 0.5	35.7 ± 1.7	0.09 ± 0.01
Hypo Adrenal Cortical	With Weight Loss	5	6	412 ± 5	400 ± 6	14.5 ± 0.5	12.4 ± 3.2	0.08 ± 0.01
	With Weight Gain	5	6	357 ± 9	367 ± 7	13.4 ± 0.6	18.2 ± 3.6	0.16 ± 0.01
Females								
Controls	Diestrus	6		241 ± 7		7.5 ± 0.7	60.3 ± 2.8	0.22 ± 0.01
	Estrus	4		236 ± 9		8.6 ± 0.3	72.0 ± 6.3	0.23 ± 0.02
Hyper Adrenal Cortical	ACTH 10U./day	5	12	248 ± 5	253 ± 8	10.0 ± 0.5	142.4 ± 28.7	0.18 ± 0.01
	Cortisone Acetate, 1.5 mg./day	4	12	268 ± 5	275 ± 5	9.2 ± 0.3	54.2 ± 3.6	0.20 ± 0.01
	Cortisone Acetate, 3.0 mg./day	4	12	289 ± 2	270 ± 6	8.5 ± 0.1	40.8 ± 3.2	0.19 ± 0.01
Hypo Adrenal Cortical	6 days Post-op.	5	6	242 ± 3	243 ± 4	8.2 ± 0.3	6.0 ± 1.3	0.22 ± 0.003
	12 days Post-op.	3	12	252 ± 3	265 ± 2	9.7 ± 0.1	15.0 ± 0.9	0.21 ± 0.01

had high blood levels of both ACTH and corticosterone. Another group was treated for the same period with cortisone acetate. These animals showed the expected adrenal atrophy, and presumably had low blood levels of ACTH and high blood levels of cortisone. As shown in Figures 1 and 2, the hyperadrenalcortical state, however produced, did not cause an increase in total hepatic capacity to inactivate corticosteroids in any instance. In fact, a substrate depression of the Δ^4-steroid hydrogenase activity was revealed (Fig. 1, ACTH and cortisone groups; Fig. 2, 10.4 mg./kg./day cortisone group).

In males, the ACTH treatment caused a 30% diminution of total hepatic activity for the reduction of ring A of corticosterone. The treatment with cortisone caused a 26% loss of activity for the metabolism of corticosterone, and a 24% loss for the metabolism of cortisone itself (not shown). In females, neither the ACTH treatment, nor the 1.5 mg./day dose of cortisone (5.6 mg./kg./day) affected the enzymatic activity in the liver for the inactivation of either corticosterone or cortisone. At higher doses (10.4 mg./kg./day) cortisone caused a 14% loss of hepatic activity for the reduction of corticosterone, and a 36% loss for ring A reduction of cortisone (not shown). The dose of cortisone acetate required to reveal the substrate depression of hepatic activity was slightly more than 2 times greater in the

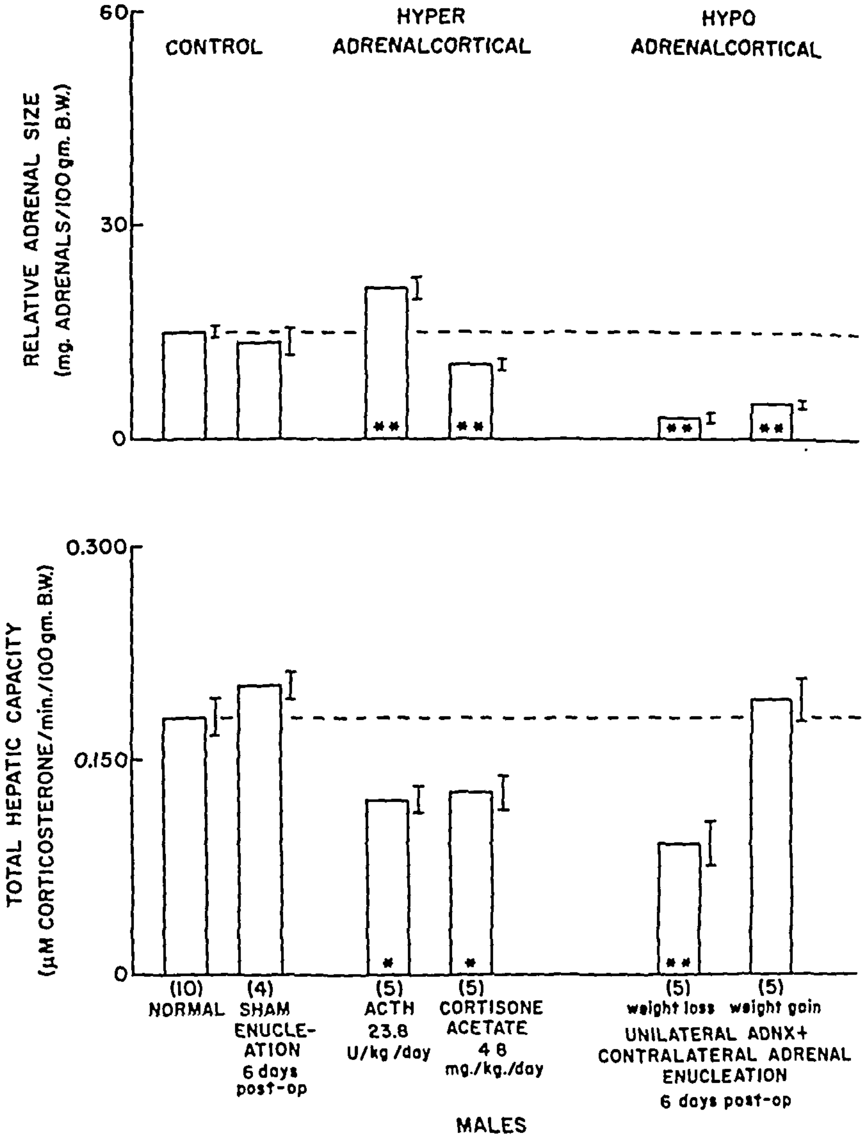

FIG. 1. Derived data showing effects in males of variations in adrenal cortical state on the hepatic capacity for reduction of ring A of corticosterone *in vitro*. Figures in parentheses indicate number of animals studied. Lines beside bars indicate standard errors of the means. The data from young adult and adult control groups were pooled. (*) indicates that the difference between the experimental value and the control value is significant at the 5% level; (**) indicates significance at the 1% level.

female (10.4 mg./kg./day) than in the male (4.8 mg./kg./day), presumably because of the greater capacity of the female to inactivate adrenal cortical hormones (10).

The acute hyperadrenalcortical state produced in four male rats by intravenous infusion of corticosterone at approximately 2 µg./min. for 4 hours also failed to cause any increase in total hepatic capacity for steroid ring A reduction. In fact, a 30% depression of activity by the substrate was observed ($P<0.01$). The control saline infusion of 3 rats had no effect on the Δ^4-steroid hydrogenase activity of the liver.

Hypoadrenalcortical groups

In males and females the hypoadrenalcortical state was achieved by complete removal of one adrenal, and removal of all but a fragment of capsule of the opposite adrenal. The enucleation was done alternately on right

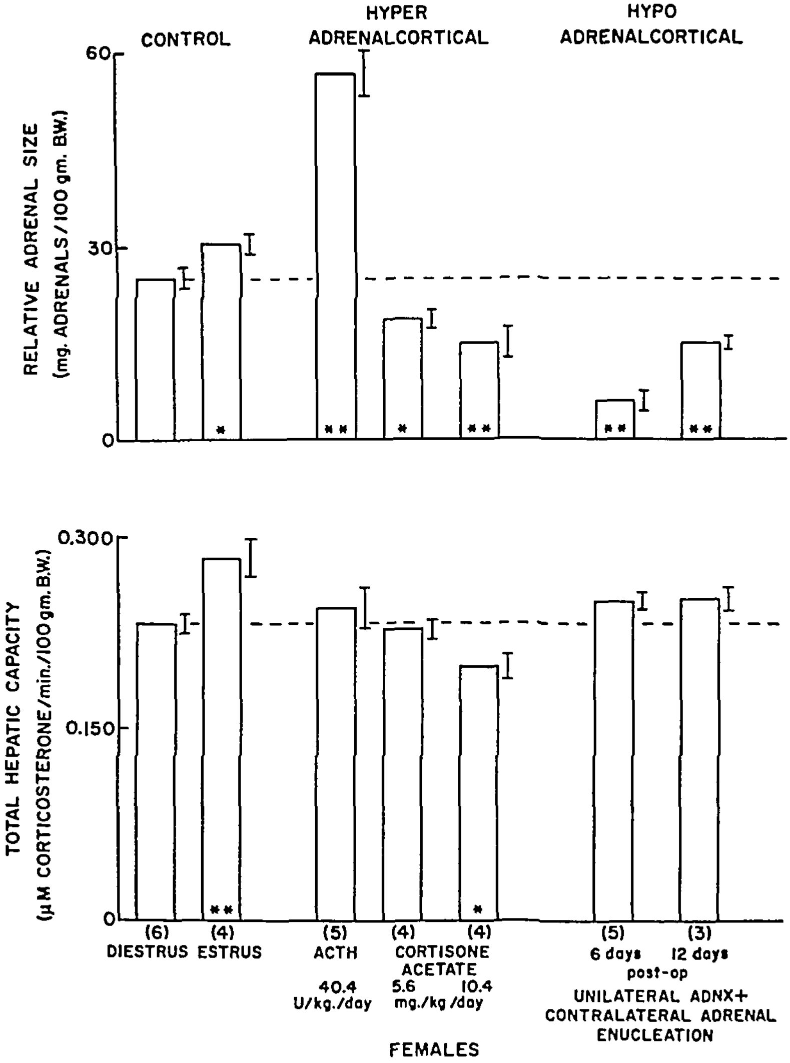

FIG. 2. Derived data showing effects in females of variations in adrenal cortical state on the heptatic capacity for reduction of ring A of corticosterone *in vitro*. Figures in parentheses indicate number of animals studied. Lines beside bars indicate standard errors of the means. (*) indicates that the difference between the experimental value and the diestrus control value is significant at the 5% level; (**) indicates significance at the 1% level.

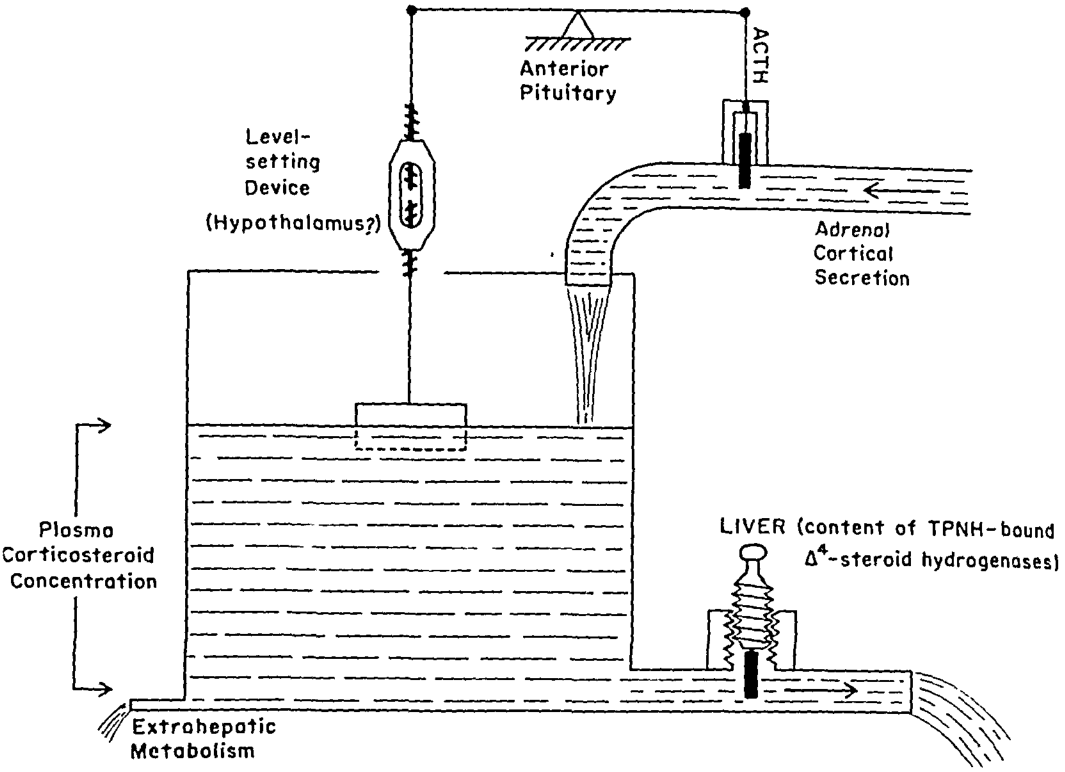
ACTH
Anterior Pituitary
Level-setting Device
(Hypothalamus?)
Adrenal Cortical Secretion
Plasma Corticosteroid Concentration
LIVER (content of TPNH-bound Δ^4-steroid hydrogenases)
Extrahepatic Metabolism

FIG. 3. Hydraulic analogue of the hepatic control of adrenal cortical secretion rate. The hepatic-anterior pituitary-adrenal cortical system can be represented schematically as a single capacity plant with proportional input regulation and proportional output self-regulation (via the first order reaction kinetics *in vivo*) at each setting of the independently variable output regulator. The instantaneous rate of outflow (inactivation), to which the rate of inflow (secretion) will be matched, is determined both by the setting of the output regulator (the total hepatic content of TPNH-bound Δ^4-steroid hydrogenases) and by the fluid level in the system (the concentration of corticosteroids in the blood).

The time constant of such a system is derived from integration of two first order differential equations, one for the inflow relation and one for the outflow relation, and is given by:

$$T = \frac{V}{S + C}$$

where T is the time it takes to diminish by 63% $[1-(1/e)]$ a deviation of plasma corticosteroid concentration from the mean value; V is the volume of distribution of adrenal cortical hormones at plasma concentration; S is the sensitivity of the input regulator; and C is the hepatic content of TPNH-bound Δ^4-steroid hydrogenases.

The liver will therefore have a controlling influence on both the rate of adrenal cortical secretion and the time required to correct an alteration in plasma corticosteroid concentration, regardless of the absolute value of the concentration. In addition, the liver may also influence the mean regulated level of corticosteroids in the blood. In the hydraulic analogue, the mean level (P_m) is given by:

$$P_m = \frac{q_{i\,max}}{S + C}$$

where $q_{i\,max}$ is the rate of inflow which would be observed if the level were zero. Hence, and left sides. These hypoadrenalcortical animals required no therapy, and the regeneration of the adrenal tissue followed the course described by Ingle (26). *Total adrenalectomy was rejected as an experimental test of the* effects of decreased adrenal cortical secretion rate on steroid metabolism by liver because prolonged absence of adrenal cortical secretion does not occur physiologically.

The assays were performed 6 days after the surgical removal of most of the adrenal tissue. At that time, only 26% and 42% of the normal amount of cortical tissue was present in two groups of males, and only 26% was present in the females, assuming that the medulla represents 9.2% of the total weight of the normal male adrenal and 6.8% of the total weight of the normal female adrenal (27). The results in the two groups of hypoadrenalcortical males are shown in Figure 1. In the male group which received the more extensive removal of adrenal tissue, all the animals lost weight during the 6-day post-operative period. In these males, which apparently had severe adrenal cortical insufficiency, there was a significant loss of hepatic capacity to inactivate corticosterone by ring A reduction (Fig. 1, hypoadrenalcortical group on left). In the second group, however, with somewhat less extensive loss of adrenal tissue there was no change in hepatic steroid metabolism (Fig. 1, hypoadrenalcortical group on right), and every one of these animals gained weight during the 6-day post-operative period, although at a slower than normal rate. A similarly prepared group was followed for 12 days after operation (not shown). Further adrenal regeneration was found, as described by Ingle (26), suggesting that the concentration of ACTH in blood was still elevated during the 6th to 12th day because of persisting low plasma corticosteroid concentration. The continuing hypertrophy of the adrenals indicates that all the animals assayed at 6 days were really in a hypoadrenalcortical state.

Sham operations consisting of bilateral excision of pieces of perinephric fat were without effect on either adrenal size or hepatic metabolism of corticosterone (Fig. 1, controls).

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the greater the outflow capacity, the lower the mean regulated level. Changes in the mean regulated level, as a result of changes in outflow capacity, are known as load-errors. In the biological system we have shown that as the hepatic capacity (C) increases, the adrenals hypertrophy, and so compensating changes in $q_{i\,\max}$ may minimize the effects of changes in hepatic capacity on the mean plasma concentration of adrenal cortical hormones. Thus, the load-error effect in the biological system may be much less than the hydraulic analogue would indicate.

The high ACTH concentration in the blood of adrenalectomized animals is further increased when the animals are exposed to stressful stimuli (37). Such an effect in animals with zero corticosteroid concentration must have been brought about by some independent variable other than the liver (e.g., hypothalamic excitation). The additional independent variable is represented in the figure by the turnbuckle.

The hydraulic analogue is based upon Smith (29).

Similar results were obtained in the females made hypoadrenalcortical in the same manner, by surgical removal of most of the adrenal tissue, followed by 6 to 12 days of recovery and partial adrenal cortical regeneration. As shown in Figure 2, the hypoadrenalcortical state in females had no effect on the capacity of the liver to inactivate corticosterone by ring A reduction. Considerable adrenal regeneration occurred between the 6th and 12th day, and so it seems likely that the 6-day group was indeed in a hypoadrenalcortical state at the time of assay.

Partial hepatectomy

The results of all these direct attempts to simulate wide variations in adrenal cortical function indicate that changes in the rate of adrenal cortical secretion do not cause parallel changes in the capacity of the liver to inactivate corticosteroids.

TABLE 2. EFFECT OF REMOVAL OF 65% OF THE LIVER ON THE WEIGHT OF THE ADRENAL GLANDS. SPLENECTOMY WAS USED TO CONTROL THE EFFECTS OF ETHER ANESTHESIA AND SURGICAL TRAUMA ON ADRENAL WEIGHT

Group	Body wt. at operation (gm.)	*Wt. tissue* removed (gm.)	24 hours post-op.		48 hours post-op.		72 hours post-op.		96 hours post-op.	
			Liver wt. (gm.)	Adr. wt. (mg.)	Liver wt. (gm.)	Adr. wt. (mg.)	Liver wt. (gm.)	Adr. wt. (mg.)	Liver wt. (gm.)	Adr. wt. (mg.)
Females										
Splenectomy Controls	240 ± 6.5 (5)	0.8 ± 0.08 (5)	7.4 (1)	66.8 (1)	9.2 (1)	72.4 (1)	9.5 (2)	70.2 (2)	9.7 (1)	77.6 (1)
Hepatectomy	250 ± 2.9 (5)	6.1 ± 0.73 (5)	4.4 (1)	101.8 (1)	7.2 (1)	80.0 (1)	7.8 (2)	83.2 (2)	9.1 (1)	88.1 (1)
Males										
Splenectomy Controls	280 ± 2.6 (5)	2.1 ± 0.19 (5)	11.3 (1)	56.1 (1)	11.7 ± 0.02 (4)	55.6 ± 2.0 (4)				
Hepatectomy	281 ± 7 (5)	6.9 ± 0.32 (5)	3.8 (1)	41.4 (1)	6.3 ± 0.4 (4)	41.3 ± 1.1 (4)				

We then attempted the converse experiment of testing whether direct alterations in the amount of liver tissue would lead to parallel changes in adrenal size. For this purpose male and female rats of the caesarian-delivered Sprague-Dawley strain (Charles River Laboratories) were subjected to surgical removal of approximately 65% of their total liver tissue. They were studied between 24 and 96 hours after operation. The weights of the regenerating liver and of the adrenals were measured and compared to the liver and adrenal weights of animals of the same size which had received total splenectomy as a control operation at the time of the hepatectomy in the others. The results are shown in Table 2.

The adrenals of partially hepatectomized males were atrophic, compared to those of their sham-operated controls ($P < 0.01$) 48 hours after operation, in spite of the fact that more surgical trauma was involved in the hepatectomy than in the splenectomy. Approximately three times more liver tissue than spleen tissue was removed. In the females the effect of partial hepatectomy on adrenal size could not be examined, because the liver regenerated almost completely while the adrenals were still hypertrophied as a result of the stress of the operation. The results in the males, however, show that primary changes in hepatic capacity for inactivation of adrenal cortical hormones can cause parallel changes in adrenal size.

Effects of stress

The demonstration of a very rapid substrate depression of the enzymatic inactivation of corticosteroids by ring A reduction during the four-hour intravenous infusion of corticosterone, suggested to us that this effect of corticosteroids on the liver might reinforce the hyperadrenalcortical state of stress at a time when the plasma concentration of adrenal cortical hormones is high. We studied various types of experimental conditions which are commonly thought to provoke "stress" responses in rats (cold, fasting, trauma, ether anesthesia, exercise) and found that in most instances the ability of the liver to metabolize steroids by ring A reduction was severely diminished. In no case was it increased. These results will be reported in detail elsewhere.

DISCUSSION

The results of the present experiments show that changes in plasma conconcentration of ACTH or adrenal cortical hormones do not cause parallel changes in the hepatic capacity for the inactivation of corticosteroids by ring A reduction. Only in the extreme case of the male group with adrenal cortical insufficiency and weight loss was the change in steroid metabolism in the same direction as the change in adrenal cortical state. Therefore, the close parallel relationship between adrenal size and hepatic metabolism of adrenal steroids previously described does not represent a secondary response of the liver to primary changes in adrenal cortical secretion rate. In contrast, however, independent alterations of hepatic capacity to inactivate adrenal cortical hormones can cause secondary changes in adrenal size. The adrenals of partially hepatectomized male animals were significantly smaller than those of their sham-operated controls (Table 2). From these data we have concluded that the many examples of parallel changes in adrenal size and hepatic capacity to inactivate corticosteroids demonstrate an hepatic control of adrenal cortical secretion rate.

The controlling influence of the liver on adrenal cortical function could be mediated by the mechanism proposed in the negative-feedback hypothesis. According to this hypothesis, the release of ACTH from the anterior pituitary is inhibited by adrenal cortical hormones (2, 3). As a consequence of this inhibition, changes in the plasma concentration of corticosteroids cause opposite changes in adrenal cortical secretion rate, so that the concentration tends to be restored to its initial level. Concentration changes brought about by the liver could signal compensating changes in adrenal cortical secretion rate through such a mechanism, as shown schematically in Figure 3. After hypophysectomy, the controlling influence of the liver on adrenal cortical function appears to be lost. Hypophysectomy in the rat prevents the adrenal hypertrophy of hyperthyroidism (29, 30), and abolishes the sexual dimorphism of the adrenal glands (31), both of which we have considered to be examples of the controlling influence of the liver on adrenal cortical secretion rate.

The extraordinarily close correlation ($r = 0.97$) between adrenal size and the capacity of the liver to reduce the Δ^4 group of corticosteroids (9) suggests that the negative-feedback control very effectively matches the mean input of adrenal cortical hormones to any given output over a wide physiological range of steroid inactivation rates. Through this feedback mechanism, variations in the hepatic capacity for adrenal cortical hormone inactivation may regulate the rate of ACTH release, as well as the rate of secretion of adrenal cortical hormones. Therefore, in any physiological state in which the liver maintains this controlling influence on adrenal cortical secretion rate, no valid estimate of changes in plasma corticosteroid concentration can be made solely from changes in plasma ACTH concentration, adrenal size, or adrenal cortical secretion rate.

Alterations in the hepatic capacity to inactivate adrenal cortical hormones may affect the amplitude of the hunting of the plasma corticosteroid concentration about a mean value. For example, ACTH release may be influenced by the rate of change of plasma corticosteroid concentration as well as by the absolute magnitude of the change, just as the magnitude of the change in stimulus intensity required to elicit a response from thermal and stretch receptors is inversely related to the rate of the change (32, 33). If the rate of hepatic inactivation of corticosteroids is diminished, a substantial decrement in plasma corticosteroid concentration might occur before the steroid inhibition of ACTH secretion is decreased, and adrenal cortical secretion begins to re-establish the initial concentration. Conversely, if the rate of steroid inactivation is elevated, a very small decrement in concentration might serve to provoke the compensatory response from the anterior pituitary and adrenal cortex. This proposed inverse relationship between hepatic capacity for steroid inactivation and the amplitude of variation of the plasma concentration of corticosteroids about a mean value may explain the difference in variability of plasma corticosterone levels found among groups of male, diestrus-female, and estrus-female rats (34). The coefficients of variation about the means were 34.8%, 14.8%, and 8.6%, respectively.[4] The hepatic capacities for inactivation of corticosterone which we have measured in similar groups are 0.17, 0.23, and 0.28 μM/minute/100 gm. body weight, respectively (Figs. 1 & 2).

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[4] We derived the coefficients of variation from the data presented as means and standard errors of the means. An "F" test showed that all differences among the standard deviations of the means of the three groups were significant ($P < 0.01$) although the means themselves were almost identical and the groups with the smallest variability contained the fewest animals.

Although our results indicate that the liver is a principal determinant of ACTH release and adrenal cortical secretion rate in normal animals, other independent determinants of ACTH release may supersede the hepatic regulation of adrenal cortical function under abnormal conditions such as stress. These other influences on ACTH release (e.g. hypothalamic excitation) can cause sustained elevations of adrenal cortical secretion rate which are independent of any changes in hepatic metabolism of the steroid hormones (Table 2, females). The failure of either ACTH or adrenal cortical hormones to increase the capacity of the liver for corticosteroid inactivation indicates that any metabolic effects following such sustained elevations of corticosteroid inflow cannot be abolished by the liver. Increased hepatic capacity for corticosteroid inactivation is therefore not involved in the unexplained escape of animals from the initial sodium and water retention, observed under certain conditions, during treatment with large doses of ACTH or adrenal cortical hormones (35, 36).

Acknowledgment

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NOTES AND COMMENTS

THE EFFECT OF ACTH ON ADRENAL ASCORBIC ACID IN LAYING HENS

ABSTRACT

In experiments on the effect of ACTH-administration to one-year old laying hens and three-month old pullets, the following results were obtained:

In one-year old laying hens adrenal ascorbic-acid was depleted by 57.0% as compared to their controls. However, no effect of ACTH could be elicited in three-month old pullets.

These results suggest, therefore, that the failure of other workers to obtain a depletion in adrenal ascorbic-acid upon ACTH-treatment may be attributed to the age factor.

Ascorbic acid depletion in the adrenals of animals as a result of corticotropin administration appears to be reasonably well established (1, 2). This fact is explained by the biogenesis of the corticosteroids. Several workers reported that in this respect, the behavior of the avian adrenal gland is quite different from that of other animals, since they failed to observe ascorbic acid depletion in the adrenals of chickens (3, 5, 4), ducks (6), and quails (7) after ACTH administration.

The particular status of birds concerning the elicitation of this reaction cannot be explained on the basis of the slightly different histological appearance of the fowl's gland; the authors, therefore, found it worthwhile to reinvestigate the problem.

MATERIALS AND METHODS

Twelve one-year old White Leghorn laying hens, and twelve three-month old pullets of the same breed were used in these experiments. The birds were divided into 4 equal groups, i.e. six chickens per group. Groups I and II consisted of laying hens, and Groups III and IV of pullets. Each bird in Groups I and III was given a 0.5 ml. intravenous injection of 10 I.U. of ACTH, while the birds in Groups II and IV served as controls, receiving each 0.5 ml. of saline intravenously. The ACTH used was Armour "ACThar" corticotropin, lot number S/43901. All injections were performed exactly one hour before sacrificing the birds for the subsequent removal of their adrenals. The birds were killed by cervical dislocation, and the two adrenals of each bird were weighed together, and their combined ascorbic acid was determined. The method used for ascorbic acid determination was that of Roe and Kuether (7), using Norit as an oxidizing agent. Colorimetric readings were made on a "Hilger" electrophotometer.

RESULTS AND COMMENTS

As may be seen from the table, the average absolute amount of adrenal ascorbic acid in the non-treated laying hens (Group II) was approximately 30% lower than that of the pullets. The ascorbic acid concentration of the pullets' glands was twice that of the hens, due to the smaller weight of the pullets' adrenals. ACTH injections in the laying hens (Group I) caused an average depletion of 57% in their adrenal ascorbic acid content. At the same time, no effect of ACTH treatment on adrenal ascorbic acid depletion could be detected in the 3-month old pullets.

In view of the lack of effect of ACTH injections on the adrenal ascorbic acid of immature chickens, and in view of the positive results obtained with older birds in our experiments, the following conclusions may be drawn:

1. The failure of other workers to observe a depletion of adrenal ascorbic acid in birds following ACTH injections may be attributed to the age factor, as their experiments

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EFFECT OF ACTH ON ADRENAL WEIGHT AND ASCORBIC ACID CONTENT

	Mean adrenal weight (mg.)	Mean ascorbic acid content (mg. %)	Mean ascorbic acid concentration (mg. %)
Group I ACTH-treated Laying Hens	135 ± 11.00	95 ± 7.40	70 ± 5.00
Group II Saline-treated Laying Hens	138 ± 14.00	222 ± 19.00	161 ± 18.20
% Difference between Groups I and II		57%	56%
Group III ACTH-treated Pullets	94 ± 3.00	309 ± 13.20	329 ± 11.60
Group IV Non-treated Pullets	91 ± 2.40	305 ± 2.78	335 ± 7.00
% Difference between Groups III and IV		1.3%	1.9%

were carried out on 2-day old chicks (3), 37-day old chicks (4), 6- to 10-week old cockerels (5), 23-day old ducks (6), and 12-week old quails (7).

2. In older birds, the adrenals respond to ACTH treatment with the same sensitivity as mammalian adrenals, when judged by the depletion of ascorbic acid content. Similarly, age differences have been observed in the chicken in connection with other phenomena linked to adrenal activity. Thus, while a single injection of adrenal cortical extract (ACE) in the adult fowl caused a transient lymphopenia, and leucocytosis (9), ACE in the chick embryo caused no change in lymphocytes, but a rise in polymorphonuclear cells (10). ACTH in the 5-day old chick had no effect on the number of eosinophils (11).

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INFLUENCE OF ANDROGENS ON ENZYMES OF THE KREBS CYCLE[1]

ABSTRACT

The concentration and total fumarase activity of mouse but not rat or guinea pig liver was decreased by castration and maintained or restored to normal by testosterone administration. The activities of succinic, malic and isocitric dehydrogenases of the liver were not altered in any of the species. The concentration of activity of none of the enzymes was altered in the kidney, heart or muscle. Thus, those tissues which changed in weight after castration and androgen administration exhibited a change in total enzyme activity in direct proportion with the change in weight of the tissue.

Androgens are able to stimulate the synthesis of new tissue in many organs of the body (1) and also increase the utilization of body fat (1, 2, 3) without an apparent increase in energy metabolism (1). It seemed important, therefore, to determine whether the activity of any of the enzymes of the energy producing Krebs Cycle were altered during these androgen-induced changes in metabolism.

Animals: The rats (Sprague-Dawley) were maintained in individual metal cages, the mice (National Laboratories, Creve Coeur, Mo., or Stokeley-Peterson Co., Madison, Wis.) were kept in groups of 3 or 4 in glass jars containing wood shavings, and the guinea pigs (Hartley strain, Tumblebrook Farms) were kept in groups of 3 to 4 in metal cages with screen bottoms. The rats and mice were fed ad libitum the Rockland Mouse diet and the guinea pigs received the Purina Rabbit Checkers or Superior Rabbit pellets with supplements of 25 mg. of Vitamin C and 30–40 gm. carrots three times per week. The artificial light was regulated at 12 hours per day. The temperature for the rats and mice was maintained at 25–27° C and that for the guinea pigs at 22–23° C.

Castration and subcutaneous implantation of pellets of androgens[2] were carried out under ether anesthesia.

Enzyme determination: The guinea pigs and rats were stunned by a blow on the head and then bled to death by cutting the blood vessels of the neck. The mice were killed by dislocating the spine at the base of the skull and cutting the blood vessels of the neck. The tissues were immediately removed, weighed and an appropriate aliquot placed in ice-cold redistilled water for homogenization in Potter-Elvejhem type all glass homogenizers.

The methods for the determination of succinic dehydrogenase (4, 5), malic dehydrogenase (5), isocitric dehydrogenase (6) and fumarase (7) were as reported. The succinic dehydrogenase experiments with mice were repeated with the Warburg procedure (5) with essentially the same results.

RESULTS

Organ Weights: The changes in organ weights due to castration and androgen treatment were as expected (1, 8).

Fumarase: Mice. Castration did not significantly alter the concentration of fumarase activity in the heart or the kidney of the mouse (Fig. 1). Thus, the activity of the enzyme changed in direct proportion with the changes in weight of these two organs. The activity of fumarase in the liver (Fig. 1), on the other hand, showed a definite decrease in concentration within 10 days after castration and remained low during the period of castration. Furthermore, castration at an older age, resulted in a prompt decrease in the concentration of activity. The implantation of a pellet of testosterone at the time of castration or 60 days later maintained and restored respectively the activity of the enzyme.

Rats and Guinea Pigs: The concentration of fumarase activity of the liver, kidney and heart of rats and guinea pigs was not changed after castration or various doses and duration of androgens.

Malic, succinic and isocitric dehydrogenases: The activities of these enzymes in the

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[1] This investigation was supported by research grants, C-1954 and CY-3566, from the National Cancer Institute of the Public Health Service.

[2] The androgens were generously provided by Syntex and Ciba Pharmaceutical Products, Inc.

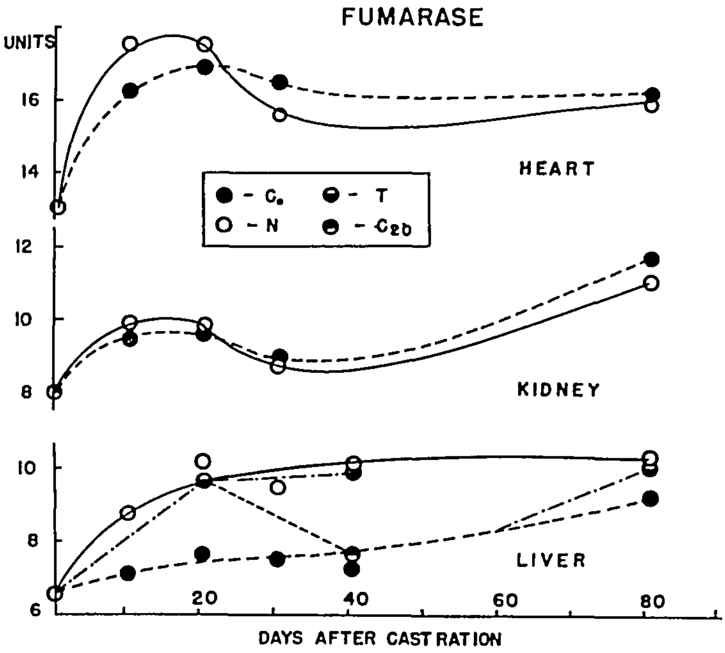

FIG. 1. The effect of castration, testosterone and age on the concentration of fumarase activities in the tissues of the mouse. C_0 = Castration at 35–40 days of age; C_{20} = Castration at 55–60 days of age; T = 15 mg. pellet of testosterone implanted subcutaneously 21 days before autopsy. The units are per mg. wet weight of tissue; 1 unit = a change of 0.01 O.D./min. at 240 mμ. (7).

liver, kidney and heart of the mouse, rat and guinea pig were not affected by castration or androgen administration. Furthermore, the malic and succinic dehydrogenases were not altered in the temporal muscle of the guinea pig or the gastrocnemius muscle of the rat.

Effect of Age: A progressive increase with age was evident in the activity of isocitric dehydrogenase of the mouse tissues especially in the heart and an initial increase in the fumarase activity. The other two enzymes were not studied over a sufficiently long period of time to warrant any definite conclusions.

DISCUSSION

These studies indicate two definite roles of the androgens in regulating the activities of the Krebs Cycle. The first is a general function. These enzymes change in activity in direct proportion with the decrease in protein synthesis after castration and the increase after androgen administration as seen in the kidney and heart of the mouse and rat and the temporal muscle of the guinea pig. Since most of these enzymes are present in the mitochondria, it would seem that the androgens induce synthesis of some elements of the mitochondria at the same time that they are inducing synthesis of other protein constituents of the particular tissue. Several other enzymes are known to change in direct proportion with the changes in weight of these same tissues after castration and androgen administration, e.g., the alanine-glutamic and aspartic-glutamic transaminases of the temporal muscle of the guinea pig (9) and the kidney and heart of the mouse (10), the acid phosphatase of the mouse kidney (11), and adenosine triphosphatase of the kidney and the heart of the rat and the mouse (10). Furthermore, the succinic dehydrogenase (12) and the oxygen consumption (13) of the perineal muscles of the rat also show a directly proportionate change with weight.

It is noteworthy that the decrease in fumarase activity of the liver of the mouse is prevented or restored by testosterone administration. The activity of the enzyme, however, is never increased above normal. Furthermore, the need of androgen to maintain the fumarase at normal activity is peculiar to the mouse liver. Not only the other tissues but also the liver of the rat and guinea pig show no tendency to require androgen for the maintenance of normal fumarase activity.

The reports (14, 15) that castration results in higher succinic dehydrogenase activity in the liver of rats cannot be reconciled with the results of this study.

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THE EFFECT OF EXCESSIVE IODINE ON THE THYROID OF THE RAT

ABSTRACT

The immediate effect of large single doses of iodide on thyroid function in the rat has been studied with the aid of I^{131} and chromatographic analysis. Organic binding of iodide is initially depressed, but the effect wears off after 16 hours presumably as the plasma iodide declines. The ratio of monoiodotyrosine to diiodotyrosine is elevated at the time of minimal binding.

Long term treatment (7 weeks) of rats with iodide results in an initial suppression of I^{131} binding in the thyroid. The effect has disappeared after 3–4 days but iodide binding falls slowly during the ensuing weeks. There is little or no evidence of hypothyroidism in these animals.

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In 1948, Wolff and Chaikoff (1) showed that the organic binding of iodine by the thyroid gland of the rat could be almost completely blocked by the administration of relatively large doses of iodide. This inhibition was related to plasma iodide levels and lasted about 17 hours. It was further shown that the amount of diiodotyrosine (DIT) formed in the gland was smaller, and the conversion of DIT to thyroxine (T_4) was considerably slower when large amounts of iodide were injected (2). However, even if a high plasma level of iodide was maintained, the blocking effect was only temporary, and the formation of significant amounts of organically bound iodide was resumed within 24 hours (3).

The recent findings (4) that the rat thyroid gland could synthesize monoiodotyrosine (MIT) under the influence of propylthiouracil (PTU) and perchlorate, but could not carry thyroid hormone biosynthesis beyond this point prompted us to investigate the nature of the small fraction of organic iodine formed when I^{131} was injected together with a large dose of nonradioactive iodide.

METHODS AND MATERIALS

Adult male hooded rats were fed on the Bruce and Parkes diet 41b (diet 41 (5) omitting cod liver oil and containing a vitamin supplement); the iodine content of the diet is not known. In order to determine the effect of a single dose of iodide each rat was given an intraperitoneal injection of 500 μg. iodide (as KI) together with 100 μc. I^{131}. All experimental groups contained 6 animals. The rats were anesthetized with ether and their thyroids were removed at 1, 2, 4, 8, 16, and 24 hours after the injection. The glands were immediately chilled in a beaker standing in dry ice. In other experiments I^{131} was administered 1 or 2 hours after 500 μg. iodide; the thyroids were removed 4 hours later.

Experiments were also carried out on rats pre-treated with iodide, administered as a 0.01% iodide in the drinking water; in this way each animal received about 1 mg. iodide per day. Thyroid analyses were performed daily for 7 days and at 2, 3, 5 and 7 weeks; each animal was injected with 500 μg. iodide and 100 μc. I^{131}, the thyroids were removed 4 hours later and chilled.

Control rats were injected with 10 μc. I^{131} alone and their thyroids were removed and analyzed at the appropriate time after the injection.

Analysis of thyroid glands

Protein bound thyroidal I^{131} was determined in the manner previously described (4). In some experiments, the radioactivity in the glands was estimated in a well-type scintillation counter before and after dialysis against running tap water. Portions of the glands were hydrolyzed with trypsin in borate buffer at pH 8.6 and the hydrolysates were chromatographed in butanol-dioxan-2N NH_3, 4:1:5 (BDA) and butanol saturated with 2N acetic acid (BAc). The distribution of radioactivity on the chromatograms was measured in an automatic strip counter.

Determination of thyroidal I^{131} uptakes and thyroid: serum iodide (T/S) ratios.

Normal rats and rats pre-treated with PTU (10 mg.), injected intraperitoneally 1 hour before the experiment, were given 0.1 μc. I^{131}; rats injected with 500 μg. iodide received 10 μc. I^{131}. One hour later the thyroids were removed and weighed, and the radioactivity in the glands and sera was measured. The thyroidal PBI^{131} and inorganic I^{131} was measured as described above.

RESULTS

Effect of a single injection of iodide

During the first 2 hours after the injection of 500 μg. iodide and I^{131}, the PBI^{131} was less than 10% of the total thyroidal I^{131}, compared with over 90% in the controls; thereafter it rose steadily and at 16 hours did not differ from that of the controls (Fig. 1).

Chromatographic analysis of thyroid hydrolysates showed that the ratio of I^{131} MIT/DIT in the treated animals was considerably higher than in the controls in the first 8 hours after injection; during this period in the experimental group, only small amounts of labelled DIT could be detected (Table 1). However, after 16 hours the I^{131} MIT/DIT had returned to normal levels. Similar results were obtained when the iodide was administered 1 hour before the I^{131}.

Table 1. Immediate effect of iodide administration on thyroidal I^{131} MIT/DIT

Time after injection (hours)	I^{131} MIT/DIT	
	Experimentals	Controls
1	4.9	0.86
2	4.2	0.92
4	4.4	0.82
8	3.7	0.53
16	0.66	0.63
24	0.57	0.63

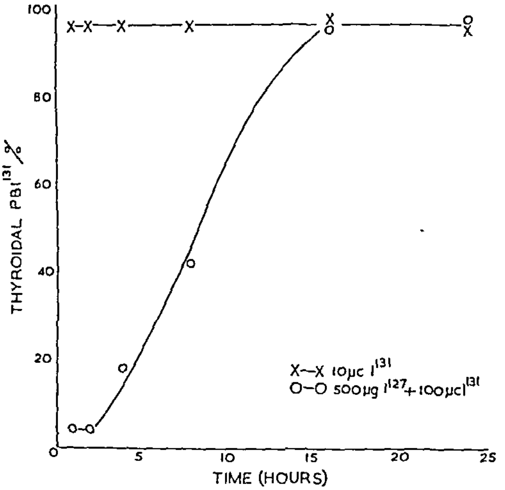

Fig. 1

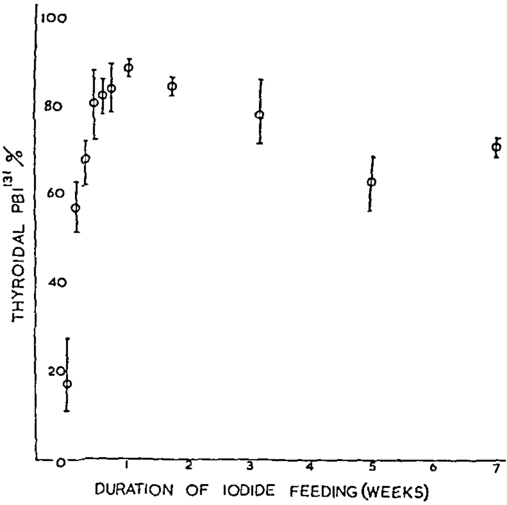

Fig. 2

Fig. 1. Effect of a single large dose of iodide on organic binding of I^{131} in rat thyroid glands.

Open circles represent PBI^{131} as a per cent of the total I^{131} in the glands of rats injected with 500 μg. iodide+I^{131}.

Crosses represent PBI^{131} in glands of control rats given I^{131} alone.

Fig. 2. Effect of chronic iodide feeding (1 mg. per day) on organic binding of I^{131} in rat thyroid glands. Brackets indicate range of value obtained in each group.

Effect of chronic iodide administration

The gradual increase in thyroidal PBI^{131} per cent found in rats fed iodide for increasing lengths of time up to 7 weeks is shown in Fig. 2.

It is evident that the depressed organic binding of iodine in the thyroid which is produced by excessive iodide administration is only temporary, since 85% of the thyroidal I^{131} was protein bound in animals which have been pretreated with iodide for 4 days. Prolonged iodide feeding, however, resulted in a gradual fall in per cent of thyroidal radioiodine present as PBI^{131}. The corresponding I^{131} MIT/DIT ratios are shown in Table 2. As in the short term experiment, this ratio was highest when organic binding of iodine was most depressed; at all times it was somewhat higher than normal.

T/S ratios

In normal rats and in those where organic binding of iodide was blocked with PTU, the T/S ratios varied between 31 to 158. The average hourly thyroidal I^{131} uptake in

TABLE 2. EFFECT OF PROLONGED IODIDE FEEDING ON THYROIDAL I^{131} MIT/DIT

Duration of treatment (days)	I^{131} MIT/DIT
1	1.25
2	1.20
3	0.60
7	0.62
14	0.82
21	0.75
35	0.92

normal animals was 3.3% of the dose, and increased to 10.6% in 24 hours. In the iodide treated rats the T/S ratio varied between 11 and 22; the I^{131} uptake was 0.21% and 0.56% of the dose 1 and 24 hours after the injection.

COMMENT

These findings are in agreement with those of Wolff and Chaikoff (1) who reported that a single excessive dose of iodide exerted an immediate depressing effect on the organic binding of iodine by the rat thyroid. This effect lasted about 16 hours, by which time the concentration of iodide in the plasma had markedly decreased. The present results have also shown that when organic binding of iodide is most depressed, the MIT/DIT ratio is very high. An elevated MIT/DIT ratio has been observed in several conditions where the thyroid might be expected to be iodine deficient (4, 6, 7) or where thyroid function was undeveloped (8), or abnormal (9); it appears surprising that this ratio should be elevated when the gland must be saturated with iodide.

In the rats pretreated with iodide the marked depression of organic binding was only temporary and the gland escaped from this effect after 3–4 days. However, the organic binding appeared to decrease slightly with continued treatment (Fig. 2) although the MIT/DIT ratios were not greatly elevated. The thyroid glands of rats that had been fed iodide water for 7–8 weeks were normal in appearance and weight. Histological examination of these glands showed only a slight increase in follicle size with minimal changes in cell height. Neither did these animals show the usual signs of hypothyroidism such as coarsening and loss of hair and the increased weight found in thyroidectomized adult rats. It appears that the rat thyroid does not respond in the same dramatic way to iodide as does the chick thyroid (10). Whether even more prolonged administration of iodide will eventually induce goiter in rats as it can occasionally do in man (11–19) is at present being investigated.

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A TEST FOR PROLACTIN USING THE HYPOPHYSECTOMIZED EFT PHASE OF *DIEMICTYLUS VIRIDESCENS*

ABSTRACT

Hypophysectomized, terrestrial efts of *Diemictylus viridescens* respond to treatment with prolactin above an estimated threshold of 0.04 I.U. by migrating to water. The protocol for conducting a prolactin assay using the eft is described and the results of an assay of highly purified prolactin are given.

In recent years a minimum dose assay method for the estimation of prolactin has been developed using the terrestrial (red eft) stage of the eastern spotted newt, *Diemictylus viridescens,* as the test organism. A description of the technique is presented here in order that its protocol be available to those interested in the problem.

Following metamorphosis, individuals of *Diemictylus* enter into the terrestrial eft phase which usually lasts for about three years. The animals then return to water and develop the laterally compressed (keeled) tail, lateral line system and olive pigmentation of the adult. Reinke and Chadwick (1) demonstrated that efts receiving implantations of whole adult pituitaries voluntarily entered water within a few days following treatment and assumed adult morphological characteristics. Following injections of 14 to 20 mg. of prolactin (Eli Lilly unidentified lot) Chadwick (2) was able to induce such a "water drive syndrome" in thyroidectomized and gonadectomized efts. However, conflicting evidence was presented by Dr. R. W. Payne (personal communication) who elicited the same reaction with various other pituitary preparations. These results may be attributed to endogenous release of pituitary hormones. In investigations utilizing hypophysectomized efts Grant and Grant (3, 4) have verified prolactin in the role of the active water drive principle, but have shown that it is not directly involved with the production of the range of morphological changes associated with the complete water drive syndrome. There is no difference in the response of efts to prolactin above the 3 μg. level (0.10 I.U.) at which all animals respond according to Grant (5), and unpublished data indicates a threshold at about 1.4 μg. (0.049 I.U.) for highly purified prolactin (C. H. Li).

METHODS

Care of test animals: Efts can be kept for several months in good condition without feeding in plastic containers or aquaria provided with earth and a few dried leaves. Their environment should be kept slightly moist but not damp. The white worm *Enchytraeus* or mutant stocks (vestigial or miniature wing) of *Drosophila* provide adequate food when necessary. For ease of handling and assurance that the newts are well removed from the water drive phase of their natural cycle, test animals should be between 40 to 80 mm. in total length and should weigh in a range from 0.5 to 1.5 gm.

Operation protocol: Following anaesthetization in 0.5 per cent chloretone animals are weighed and measured. Recording of the dorsal spot pattern is convenient for the identification

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TABLE 1. THE WATER DRIVE RESPONSE OF HYPOPHYSECTOMIZED EFTS OF *Diemictylus viridescens* TO VARIOUS DOSE LEVELS OF A HIGHLY PURIFIED PROLACTIN (C. H. LI)†

Number of animals	Average weight in gm.	Average length (mm.)	Total dose		% response	Time to water in hours
			Mg.	I.U.		
6	0.823	32.8	0.4	14	100	139 ± 32.03*
6	0.795	35.7	0.04	1.4	100	123 ± 27.21
6	0.991	38.9	0.004	0.14	100	164 ± 38.62
9	0.712	31.2	0.001	0.035	22.2	121 ± 7.80
6	0.874	35.1	0.0005	0.017	0	

* Standard deviation.

† The author is indebted to Dr. C. H. Li of the Hormone Research Laboratory, University of California, who supplied the prolactin used in these tests.

of individuals. Efts are placed on a board in a slight depression padded with cotton soaked in Ringers solution and are held in place with rubber bands. Working under a dissecting microscope, the lower jaw is depressed and the pituitary area identified through the epithelium of the roof of the mouth. It appears as a small, circular area lying at the base of the triangle formed by the diverging row of vomero-palatine teeth. Using a fine blade, microdissection knife the mucosa of the region is cut and a square is carefully chipped away in the underlying bone revealing the pituitary. The pipette used for the removal of the gland is connected to an aspirator and is provided with a small, lateral hole for the regulation of pressure. When the gland has been removed, the chip of bone is replaced. The wound heals rapidly within a period of several days. Though it is advisable to check the operation by sectioning at the termination of the experiment, complete hypophysectomy is usually indicated when the skin of the animals becomes rough and dark because of their failure to molt in the absence of TSH. Following operations the efts are subject to infections but reasonably good prophylaxis can be obtained by placing the animals in a saline solution of malachite green (1:15,000) and potassium dichromate (1:200) for several minutes. An occasional bath in Achromycin (1:100) is necessitated in some cases. In no case should listless or diseased animals be used in tests and animals should be allowed at least two weeks to come to equilibrium following hypophysectomy.

Injection protocol: Test materials (homogenates, dried preparations, etc.) are prepared in a standard amphibian Ringers and delivered from a 0.25 ml.-capacity tuberculin syringe with a 27 gauge Huber point needle. The eft is held belly up with the hind leg of one side held back against the base of the tail. The needle is introduced into the ventral surface of this leg and pushed forward beneath the skin until it passes into the peritoneal cavity just lateral and anterior to the vent. By using the leg to guide and stabilize the needle, internal injury is kept to a minimum. Although the dose is varied to suit the convenience of the experiment, the volume delivered at each injection should be held constant. Experience indicates that a 0.1 ml. injection of a solution delivered every other day is most practical, although if a single injection is contemplated, 0.2 ml. can be injected into larger newts.

Following injections animals are placed in a container (11×8×4 inches for 8 animals) provided with an inch high cross partition of lucite which serves to divide the chamber into an aquatic and terrestrial area. The latter is provided with clean, aquarium pebbles and several pieces of moistened filter paper to provide cover for the animals. A positive test for prolactin is indicated when the efts voluntarily enter water and remain submerged for at least six hours at a time. Water drive usually occurs from five to twelve days following the initial injection. When whole pituitary preparations are administered, the animals molt frequently and upon entering water progress through the water drive syndrome. Olive pigmentation appears in several weeks, while the keeling of the tail is apparent somewhat later. When pure prolactin or preparations with a very high prolactin content are used, water drive alone is elicited. In this case animals fail to molt properly, although patches of epithelium slough off after the animals have been in water for several days.

In order to obtain quantitative estimates of prolactin content, it is at present necessary to use a minimum-dose assay technique based on a threshold of 1.4 μg. (0.049 I.U.), estimated at causing water drive in 50 per cent of test animals. The red eft test has recently been applied with success on elasmobranch, teleost and primate material and should prove of value in situations where the standard pigeon crop method proves impractical. An example of a typical assay series is given in Table 1.[1]

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[1] These results were obtained in an investigation supported by a research grant, A 1786 (C 1), from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

CHROMATOGRAPHIC ARTIFACTS OBTAINED WITH SODIUM IODIDE-I^{131}

Doctor and Trunnell (1) have recently reported experiments on the effect of storage of sodium iodide-I^{131} and the action of various reducing agents on the products obtained from it during storage. From their results, doubt might be cast on the validity of quantitative aspects of I^{131} metabolism determined in humans or experimental animals since only a proportion of the I^{131} appears to be present as iodide.

It has been observed by many workers that if carrier-free sodium iodide-I^{131} is chromatographed in various solvent systems, a number of radioactive bands appear on the chromatograms. If the same sample of radioactive iodide is chromatographed with a small amount of sodium iodide I^{127}, all the bands except the iodide band disappear. This is found whether the radioactive iodide is fresh or stored. It seems therefore that the apparent breakdown of sodium iodide-I^{131} is artifactual and probably results from the absence of a sufficient number of stable iodide ions to allow it to manifest its usual chromatographic mobilities.

In biological experiments, injection of carrier-free I^{131} into the blood stream or tissues will be followed by its immediate dilution by the iodide present in the body. Further, it will quickly come into contact with tissue reducing agents. Lastly it has been shown by Leblond and Süe (2) and Murray (3) that oxidized iodine in the form of iodate is rapidly reduced to iodide in the body and taken up as such by the thyroid gland. For these reasons, it is to be expected that carrier-free I^{131} will behave as iodide in the body.

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[1] Senior Postdoctoral Fellow of the National Science Foundation.

The Endocrine Society

1959 PROGRAM OF THE MEETING OF THE ENDOCRINE SOCIETY IN ATLANTIC CITY

THURSDAY, JUNE 4, 1959

8:00 A.M.—Registration—

I. 9:00 A.M.—THYROID

Carolina Room—Chalfonte

Chairmen: *J. E. Rall and W. Parson*

1. The Effect of Trypan Blue on Thyroid Function in the Rat.
   Takashi Yamada (Introduced by Monte A. Greer).
2. The Effect of Excessive Iodide on the Thyroid of the Rat.
   Valerie A. Galton and *Rosalind Pitt-Rivers.* (Introduced by Sidney Ingbar).
3. Comparison of in Vivo and in Vitro Tissue Effects of Triiodothyronine.
   Roger L. Greif.
4. The Effect of Triiodothyropropionic Acid on Serum Cholesterol and Oxygen Consumption in Myxedema and Euthyroidism.
   Robert D. Leeper (by invitation), *Richard S. Benua, Soichi Kumacka* (by invitation), *Jacob L. Brener* (by invitation) and *Rulon W. Rawson.*
5. Respiratory Stimulation in Young Dogfish by Thyroid Hormone.
   Aubrey Gorbman and *A. W. Pritchard* (by invitation). (Invited paper—20 minutes.)

INTERMISSION

6. Idiopathic Familial Elevation of Thyroxine Binding Protein.
   William H. Beierwaltes.
7. Metabolism of Thyroxine by Human Tissue Slices.
   Edwin C. Albright and *Frank C. Larson* (by invitation).
8. Metabolic Regulation of Organic Iodinations in Thyroid Homogenates.
   George C. Schussler (by invitation) and *Sidney H. Ingbar.*
9. Protein Iodination in Sheep Thyroid Mitochondrial-Microsomal Preparations.
   Leslie J. DeGroot and *Evelyn Carvalho* (introduced by John B. Stanbury).
10. Persistent Thyroid Function After Hypophysectomy.
    O. H. Pearson, A. G. Pazianos (by invitation), *E. Greenberg* (by invitation), and *R. S. Benua.*
11. Effect of Stimulation of the Cervical Sympathetic Nerve on the Thyroidal Release of I^{131}-Labeled Hormones.
    Shiro Iino, Kazuo Shizume and *Shigeo Okinaka* (introduced by Monte A. Greer).

II. 2:00 P.M.—ADRENAL CORTICAL SYNDROMES—STEROID METABOLISM

Carolina Room—Chalfonte

Chairmen: *J. McArthur and A. Bongiovanni*

12. 6 Beta Hydroxylation of Cortisol, a Quantitatively Important Pathway of Metabolism in the Human Newborn.
    E. Colle (by invitation), *R. A. Ulstrom, J. Burley* (by invitation) and *R. Gunville* (by invitation).

13. THE URINARY EXCRETION OF THREE C-21 STEROIDS IN THE ADRENOGENITAL SYNDROME.
Alfred M. Bongiovanni, Walter R. Eberlein, and *J. Darrel Smith* (by invitation).

14. THE RENAL HANDLING OF 17-HYDROXYCORTICOSTEROIDS.
R. O. Morgan (by invitation), *A. J. Blair* (by invitation) and *J. C. Beck.*

15. THE INFLUENCE OF ACTH AND HYPOPHYSECTOMY ON THE STEROID CONJUGATING CAPACITY OF THE LIVER.
David L. Berliner (by invitation), *Charles J. Nabors, Jr.* and *Thomas F. Dougherty.*

16. DIRECT AND INDIRECT ESTIMATION OF PITUITARY ACTH RESERVES IN MAN FOLLOWING STEROID THERAPY
Donald A. Holub (by invitation), *Julian I. Kitay, Andrew G. Frantz* (by invitation) and *Joseph W. Jailer.*

17. ACTH SUPPRESSION TESTS IN PATIENTS WITH CUSHING'S SYNDROME.
Grant W. Liddle.

INTERMISSION

18. URINARY STEROID METABOLITES IN HYPOPHYSECTOMIZED WOMEN DURING ADRENAL INSUFFICIENCY AND INITIAL PHASES OF ACTH STIMULATION.
Hildegard Wilson, Mortimer B. Lipsett, and *Lillian C. Butler* (by invitation).

19. SUPPRESSION OF URINARY 17-KETOSTEROIDS IN VIRILIZED WOMEN BY ESTROGEN THERAPY.
Raymond C. Mellinger and *Richmond W. Smith, Jr.*

20. TRANSCORTIN: CHARACTERISTICS AND LEVELS IN VARIOUS CONDITIONS.
Avery A. Sandberg and *W. Roy Slaunwhite, Jr.*

21. STUDIES ON THE MECHANISM OF ELEVATION OF PLASMA 17-HYDROXYCORTICOSTEROIDS INDUCED BY ESTROGEN ADMINISTRATION IN MAN
Eleanor Z. Wallace and *Anne C. Carter.*

22. ENDOCRINE EFFECTS OF METHYLENEDIANILINE.
William W. Tullner.

23. SALT LOSING HORMONE IN HYPERKALEMIC FAMILIAL PERIODIC PARALYSIS.
R. Klein.

III. 2:00 P.M.—PARATHYROIDS: PITUITARY ADRENAL SYSTEM

Viking Room—Haddon Hall

Chairmen: *G. D. Whedon and D. Nelson*

24. ISOLATION OF PARATHYROID HORMONE FOLLOWING EXTRACTION WITH PHENOL.
G. D. Aurbach.

25. PURIFICATION OF PARATHORMONE B BY COUNTERCURRENT DISTRIBUTION.
Howard Rasmussen.

26. ACTIVATION OF PARATHYROID HORMONE BY CYSTEINE.
Alexander D. Kenny.

27. EFFECT OF PARATHYROID EXTRACT ON BONE MATRIX AND SEROMUCOID.
William P. Deiss and *Leila B. Holmes* (by invitation).

28. FURTHER EVIDENCE FOR A DUAL EFFECT OF PARATHYROID HORMONE.
John J. Canary (by invitation), *H. Allan Bloomer* (by invitation), *Gabriel G. Carreon* (by invitation) and *Laurence H. Kyle.*

29. ACTH SECRETION IN RATS WITH HYPOTHALAMIC LESIONS.
A. Brodish.

INTERMISSION

30. LIMBIC SYSTEM INFLUENCES ON THE PITUITARY-ADRENAL CORTICAL SYSTEM.
John W. Mason, Walle J. H. Nauta (by invitation), *Joseph V. Brady* (by invitation), and *James A. Robinson* (by invitation).

31. EFFECT OF DIENCEPHALIC STIMULATION ON ADRENOCORTICAL AND ADRENAL MEDULLARY SECRETION IN THE DOG.
William H. Ganong and *Alan Goldfien.*
32. ADRENAL CORTICAL AND MEDULLARY RESPONSES TO TRAUMA IN DOGS WITH ISOLATED PITUITARIES.
Richard H. Egdahl (introduced by D. M. Hume).
33. THE EARLY AND LATE EFFECTS OF HYPOTHALAMIC DESTRUCTION OR REMOVAL ON PITUITARY-ADRENAL ACTIVITY.
David M. Hume and *Benjamin F. Jackson* (by invitation).
34. ION-EXCHANGE CHROMATOGRAPHY OF PITUITARY PREPARATIONS.
Andrew V. Schally (by invitation) and *Roger Guillemin.*
35. ACTH RELEASING FACTOR ACTIVE IN THE GUINEA PIG TEST.
M. Sideman (by invitation) and H. Sobel.

FRIDAY, JUNE 5, 1959

8:30 A.M.—Registration—

IV. *9:00* A.M.—ANTERIOR PITUITARY

Carolina Room—Chalfonte

Chairmen: *E. Knobil and F. L. Engel*

36. THE CELLS OF THE HUMAN ADENOHYPOPHYSIS IN ADRENAL CORTICAL AND THYROID DISORDERS.
Calvin Ezrin and *Heidi Swanson* (introduced by R. F. Farquharson).
37. A METHOD FOR THE QUANTITATIVE BIOLOGICAL ASSAY OF ACTH IN BLOOD.
Paul L. Munson, Wolfgang Toepel (by invitation) and *Mindel C. Sheps.*
38. INCREASE IN SUGAR PERMEABILITY IN THE RAT ADRENAL PRODUCED BY ACTH.
J. Eichhorn, I. D. K. Halkerston, M. Feinstein, amd *O. Hechter* (by invitation).
39. CONTRASTING REQUIREMENTS FOR THE LIPOLYTIC ACTION OF CORTICOTROPIN AND EPINEPHRINE ON ADIPOSE TISSUE IN VITRO.
J. Earle White (by invitation), *Enrique Lopez* (by invitation) and *Frank L. Engel.*
40. EVIDENCE FOR SPECIES SPECIFICITY OF HUMAN, BEEF AND RAT ACTH DEMONSTRATED BY ASSAY IN VITRO.
Julian I. Kitay. Donald A. Holub (by invitation) and *Joseph W. Jailer.*
41. A COMPARATIVE IMMUNOLOGICAL INVESTIGATION OF PITUITARY GROWTH HORMONE FROM VARIOUS SPECIES.
T. Hayashida (by invitation) and *C. H. Li.*

INTERMISSION

42. GROWTH-STIMULATING AND OTHER EFFECTS OF HUMAN GROWTH HORMONE (LI) IN A PITUITARY DWARF.
Roberto F. Escamilla, John J. Hutchings (by invitation), *William C. Deamer* (by invitation) and *Choh Hao Li.*
43. ENDOCRINE FUNCTION IN ACROMEGALY.
George J. Hamwi and *Thomas G. Skillman* (by invitation).
44. THE INFLUENCE OF HYPOPHYSECTOMY AND GROWTH HORMONE ON OXIDATION OF PALMITATE-1-C^{14} BY THE RAT.
M. J. Franklin (by invitation) and *E. Knobil.*
45. UNESTERIFIED FATTY ACID OXIDATION RATES IN ACROMEGALY, MYXEDEMA, HYPERTHYROIDISM AND DIABETES.
Josiah Brown and *Leslie R. Bennett* (by invitation).
46. EVIDENCE FOR DIRECT ACTION OF GROWTH HORMONE (GH) ON RED CELL FORMATION.
Albert S. Gordon, Burton S. Dornfest (by invitation) and *Anthony Crusco.*

47. Factors Involved in Production of Suspensions of Free Cells Derived from Hypophyses of Rats.
Griff T. Ross (by invitation) and *Robert C. Bahn.*

1:30 P.M.—ANNUAL BUSINESS MEETING, Carolina Room, Chalfonte

V. 2:30 P.M.—ESTROGENS AND ANDROGENS

Carolina Room, Chalfonte

Chairmen: *R. Huseby and L. L. Engel*

48. The Isolation and Identification of Estrogens from Peripheral Blood with a Practical Method of Analysis.
Georg W. Oertel (by invitation), *Charles D. West,* and *Kristen B. Eik-Nes.*

49. "Free Estriol" in Blood Plasma.
Joseph C. Touchstone and *John W. Greene, Jr.* (by invitation).

50. Effect of Chorionic Gonadotropin (HCG) on Conversion of Estradiol to Estriol by Perfused Human Placenta.
Philip Troen.

51. Physiologic and Biochemical Differences between the Estrogen-Sensitive Transhydrogenase and Estradiol Dehydrogenase.
C. A. Villee, D. D. Hagerman (by invitation) and *J. M. Spencer* (by invitation).

52. The Inhibition of Placental Steroid Mediated Transhydrogenase by 2′ Adenylic Acid.
Nina Hollander (by invitation), *Joseph D. Brown* (by invitation) and *Vincent P. Hollander.*

53. Adrenal Estrogens: In Vivo Conversion of Cortisone to Phenolic Estrogens.
Edward Chang (by invitation) and *Thomas L. Dao.*

INTERMISSION

54. Estrogen Synthesis in a Male Patient with a Feminizing Adrenocortical Carcinoma and Hypertension.
C. D. West, L. F. Kumagai (by invitation), *E. L. Simons* (by invitation) and *P. E. Wall* (by invitation).

55. The Effect of Estrogen on Steroid Levels and Urine.
W. L. Harrmann, I. Schindl (by invitation), and *P. K. Bondy.*

56. The Conversion of Progesterone-4-C^{14} to 21-Hydroxylated Steroids by Normal Rat Testis Stimulated with Human Chorionic Gonadotropin.
Hernan F. Acevedo (by invitation), *Oscar V. Dominguez* and *Leo T. Samuels.*

57. Steroid 21-Hydroxylase in Mouse Testicular Interstitial Cell Tumors.
Oscar V. Dominguez, Robert A. Husby and *Leo T. Samuels.*

58. Studies of Structure-Function Relationships of Steroids: Anabolic Potency of Some Testosterone Analogs.
Herbert A. Burke, Jr. (by invitation) and *Grant W. Liddle.*

59. Influence of Androgen on Prostatic Protein Synthesis.
Wells E. Farnsworth (introduced by Grosvenor W. Bissell).

VI. 2:30 P.M.—PITUITARY HORMONES—NEUROENDOCRINE MECHANISM

Viking Room, Haddon Hall

Chairmen: *J. Everett and A. Gorbman*

THOSE REGISTERED FOR THE MEETING OF THE AMERICAN DIABETES ASSOCIATION WILL BE ADMITTED TO THIS MEETING

60. Lithosperm Inhibition of Blood Pressure Depressor Effect of Oxytocin in the Fowl.

W. R. Breneman, Marvin Carmack, Daniel Overack and *Richard Shaw* (introduced by Aubrey Gorbman).

61. PLASMA AND PITUITARY LH: DETECTION AND QUANTITATIVE BIOASSAY IN THE RAT FOLLOWING GONADECTOMY.
Albert Parlow (introduced by R. O. Greep).

62. USE OF THE RED EFT DIAMICTYLUS VIRIDESCENS IN THE ASSAY OF PROLACTIN.
William C. Grant, Jr. (introduced by Aubrey Gorbman).

63. EFFECTS OF CAPTIVITY ON PRODUCTION OF GONADOTROPHINS IN PINTAIL AND BLACK DUCKS AND RESPONSE TO EXOGENOUS HORMONES.
R. E. Phillips and *A. van Tienhoven* (introduced by S. L. Leonard).

64. OVULATION IN THE DOMESTIC FOWL.
R. D. Fraps. (Invited paper, 20 minutes).

INTERMISSION

65. OVULATION AFTER STIMULATING THE RAT HYPOTHALAMUS LATE IN DIESTRUS.
J. W. Everett, R. L. Riley (by invitation) and *C. D. Christian* (by invitation).

66. DEVELOPMENT OF PERMANENT ABNORMAL CYCLES OF BEHAVIOR IN RATS AFTER EXPERIMENTAL PITUITARY DAMAGE.
Curt P. Richter.

67. HYPOTHALAMUS AND GROWTH.
S. Reichlin.

68. TSH "REBOUND PHENOMENON" IN RATS AFTER GOITROGEN WITHDRAWAL.
S. A. D'Angelo.

69. PITUITARY HORMONE PRODUCTION AND RELEASE IN THE THYROIDECTOMIZED RAT AFTER THYROXIN ADMINISTRATION.
A. N. Contopoulos, M. E. Simpson (by invitation) and *A. A. Koneff* (by invitation).

70. SUBCORTICAL REGIONS OF THE PRIMATE BRAIN REGULATING RELEASE OF VASOPRESSIN FROM THE NEUROHYPOPHYSIS.
Joseph F. Dingman, Eduardo Gaitan (by invitation), *Akira Arimura* (by invitation) and *Robert G. Heath* (by invitation).

VII. ANNUAL DINNER, Haddon Hall Hotel

E. Perry McCullagh, President, presiding

6:30 P.M.—Cocktails, The English Lounge
Sponsored by the donors of the Awards of the Society:
Ciba, Schering, Ayerst, Upjohn and Squibb. Attendance limited to holders of dinner tickets.

7:30 P.M.—Dinner—The Vernon Room
Presentation of the 1959 Awards of the Society by Charles Kochakian:
The Fred Conrad Koch Award
The Ciba Award
Ayerst Fellowship
Schering Scholar
Upjohn Scholar
President's Address: Dr. McCullagh

SATURDAY, JUNE 6, 1959

8:30 A.M.—Registration—Stair Hall, Haddon Hall Hotel

9:00 A.M.—**OPENING SCIENTIFIC SESSION OF THE AMERICAN DIABETES ASSOCIATION WILL BE HELD AT THE HADDON HALL HOTEL IN THE VERNON ROOM. ENDOCRINE SOCIETY BADGE WILL ADMIT YOU TO THIS MEETING.**

VIII. 9:00 A.M.—ADRENAL GLAND

Carolina Room, Chalfonte

Chairmen: *M. Grumbach and F. C. Bartter*

71. STIMULATORY EFFECT OF ALDOSTERONE AND CORTISOL ON THE WORK OF THE ISOLATED RAT HEART-LUNG.
Kathyrn W. Ballard (by invitation) and *George Sayers.*

72. THE HEMODYNAMIC PARAMETER MEDIATING INCREASE IN ALDOSTERONE SECRETION.
Donald S. Gann (by invitation), *Ivor H. Mills* (by invitation) and *Frederic C. Bartter.*

73. PRELIMINARY CHARACTERIZATION OF GLOMERULOTROPIN.
Gordon Farrell, Avery D. Pratt, Jr. (by invitation), *Laurence S. Harris* (by invitation) and *Robert C. Banks* (by invitation).

74. CHANGES IN METABOLIC ACTIVITIES OF STEROID HORMONES ASSOCIATED WITH METHYLATION AND HYDROXYLATION IN RING D.
Maurice M. Pechet, Evelyn L. Carroll (by invitation) and *Anne P. Forbes.*

75. EFFECT OF WALKER CARCINOMA ON STEROID DIABETES IN RAT.
David J. Ingle (introduced by Dwight J. Ingle).

76. ACUTE EFFECTS OF ADRENALECTOMY AND HYDROCORTISONE ON GLUCOSE TOLERANCE OF DIABETIC AND EVISCERATE RATS.
O. K. Smith and *C. N. H. Long.*

INTERMISSION

77. INFLUENCE OF AGE, ADRENALECTOMY AND CORTISOL ADMINISTRATION ON THE ACTIVITY OF HEPATIC GLUTAMIC-PYRUVIC TRANSAMINASE.
Homer Harding, Fred Rosen and *Charles Nichol* (introduced by Avery A. Sandberg).

78. THE METABOLISM OF 2 C^{14} PYRUVATE IN NORMAL AND ADRENALECTOMIZED RATS.
William W. Winternitz.

79. OBSERVATIONS ON THE ROLE OF THE ADRENAL GLAND IN THE METABOLIC RESPONSE TO SURGERY.
Maureen Smith, H. A. F. Dudley, J. S. Robson and *C. P. Stewart* (introduced by Grant W. Liddle).

80. RENAL-CARDIOVASCULAR PATHOLOGY IN AGING RATS WITH AND WITHOUT EXPOSURE TO ACTH AND STRESSORS.
George F. Wilgram (by invitation) and *Dwight J. Ingle.*

81. THE EFFECTS OF ANOXIS ON ADRENAL CORTICAL AND MEDULLARY SECRETION IN THE DOG.
Alan Goldfien and *William F. Ganong.*

82. EFFECT OF ADRENALECTOMY AND CORTISONE ON RATE OF ERUPTION OF MAXILLARY INCISORS IN FEMALE RATS.
L. V. Domm, W. A. Wellband (by invitation) and *J. P. Howard* (by invitation).

IX. 2:00 P.M.—INSULIN AND DIABETES

Carolina Room, Chalfonte

Chairmen: *S. Fajans and A. Renold*

83. DIFFERENTIAL RESIN BINDING OF INSULIN IN SERA FROM INSULIN RESPONSIVE AND RESISTANT DIABETIC SUBJECTS.
Marvin L. Mitchell and *Mary E. O'Rourke* (by invitation).

84. SERUM INSULIN-LIKE ACTIVITY IN PATIENTS WITH PANCREATIC ISLET-CELL TUMORS
Yves M. Dagenais (by invitation), *Albert E. Renold, Donald B. Martin* (by invitation) and *Vilma Lauris* (by invitation).

85. Role of Individual Organs in Carbohydrate Metabolism in Intact Normal Dogs.
Dorothy H. Henneman, William Shoemaker (by invitation) and *William Walker* (by invitation).

86. Hepatic Glucose Metabolism in Two Forms of Spontaneous Hyperglycemia.
Gaston R. Zahnd, Jean Christophe and *George Yerganian* (introduced by Albert E. Renold).

87. Carbohydrate Metabolism in Amphibians and Reptiles.
Malcolm R. Miller (Introduced by Frank L. Engel). (Invited paper, 20 minutes.)

INTERMISSION

88. On the Mechanism of the Blood Glucose Lowering Action of Insulin.
N. Altszular, A. Dunn (by invitation), *R. Steele, D. T. Armstrong* (by invitation), *J. S. Bishop* (by invitation) and *R. C. de Bodo.*

89. An Insulin Effect on Peptide Synthesis Independent of Glucose or Amino Acid Transport.
Ira G. Wool and *M. E. Krahl* (by invitation).

90. Comparison of Epinephrine and Insulin on Adipose Tissue Metabolism.
George F. Cahill, Jr., Bernard Leboeuf (by invitation), and *Robert Flinn* (by invitation).

91. The Action of Insulin, Tolbutamide and Hypoglycin A on the Adipose Tissue of Rats.
Thomas C. Smith, Lena Will (by invitation), and *Ira Ringler.*

92. Relation of Insulinogenesis to Pancreatic B Cell Acid and Glucose-6-Phosphatase.
Sydney S. Lazarus (introduced by Bruno W. Volk).

93. Effect of Glucagon on Hepatic Blood Flow and Metabolism in Unanesthetized Dog.
W. C. Shoemaker and *T. B. Van Itallia* (introduced by Jay J. Gold).

TO BE READ BY TITLE

See Abstracts 94–170

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EFFECTS OF ADRENAL CORTICAL STEROIDS ON CALCIUM METABOLISM

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ABSTRACT

Radioactive calcium (Ca^{45}) was used to investigate the effects of hydrocortisone and prednisolone on calcium metabolism in the rat. It was found that the absorption of Ca^{45} by the intestines was unimpaired by the administration of hydrocortisone. The administration of hydrocortisone or prednisolone to rats recently injected with Ca^{45} resulted in an increased urinary radiocalcium. This increase varied with the dosage, potency, and persistence of the steroid in the body. The uptake of Ca^{45} by the femurs of both normal and nephrectomized rats was inhibited by the administration of hydrocortisone. Inanition resulted in a very slight inhibition of Ca^{45} uptake by bone. Hydrocortisone exerted no observable mobilization on total bone calcium.

IT HAS been recognized for many years that the adrenal cortical hormones have an effect upon calcium metabolism. It is now generally accepted that the osteoporosis seen in Cushing's syndrome is the result of excess endogenous adrenal cortical secretion (1, 2). With the advent of the synthetic adrenal steroids, there was no reason to expect that their effects on calcium metabolism would be different from those of the hormones of endogenous origin. Indeed, hypercalciuria, spontaneous fractures and osteoporosis have been frequently observed after long-term glucocorticoid therapy (3–10). The administration of adrenal glucocorticoids to rats also results in hypercalciuria, the magnitude of which is dependent upon the dosage and potency of the steroid (11). However, fractures as a result of steroid treatment have been observed only in man. In the experiments to be reported, radioactive calcium was used in attempts to clarify the mode of action of the glucocorticoids upon calcium metabolism in the rat.

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MATERIALS AND METHODS

General

Albino male rats of the Holtzman strain weighing between 160 and 250 gm. were used in all experiments. The rats were fed Purina chow or a calcium-free diet (12). In the three experiments in which urines were collected for only 24 hours, the rats were fasted but 10% glucose was added to their drinking water (distilled) as a source of calories and to insure adequate urine volumes. Analyses were carried out on individual urines although the data are expressed as the averages of the individual urines. The radioactive calcium (Ca^{45})[2] was given intraperitoneally except in the experiment where the intestinal absorption of Ca^{45} was measured. Radioactivity measurements were determined on suitable aliquots of urine and on the ashed fecal matter or bone after the ash was dissolved in 1N HNO_3. The aliquots were plated out in stainless steel planchets, air-dried (overnight) and then further dried in an oven at 80° C, for twenty-four hours. All samples were counted in a flow-gas counter for a sufficient length of time to insure a statistical accuracy of 1%. The usual corrections for background, self-absorption and radioactive decay were made.

The steroids were suspended in Merck's Aqueous Vehicle #1 and were administered orally, intraperitoneally or subcutaneously. All control rats received the same volume of Aqueous Vehicle #1 as the steroid treated animals. Whenever the steroids were administered for a week or more, subcutaneous injections of penicillin and streptomycin were given to each rat (control and treated) three times weekly to prevent infection.

Total urinary and bone calcium determinations were made with a Wechselbaum-Varney flame photometer (13).

Three types of control rats were used:

(1) *Ad libitum* feeding controls.
(2) Pair-fed controls i.e., these rats received exactly the same amount of food as did their *ad libitum* feeding mates which received hydrocortisone.
(3) Pair-weighed controls i.e., the food intakes of these rats were adjusted daily in order that their final body weights would be equal to those of the treated rats.

An analysis of variance was performed on the data of each experiment.

Specific

In the experiment in which the absorption of orally administered Ca^{45} was determined, the steroid treated rats received 2 mg. of hydrocortisone/100 gm. body weight for 7 days. During this time they were on a calcium-free diet (12). Twenty-four hours before being sacrificed, each rat received *per os* 6 μc of Ca^{45} and 1.4 mg. of calcium lactate/100 gm. body weight and urine collections were begun.

In another experiment the effect on urinary radiocalcium of a single oral dose of steroid was compared with that following a single intraperitoneal injection. Forty-eight hours prior to steroid administration each animal received 10 μc. of Ca^{45}/100 gm. Urine collections were begun immediately following treatment with the steroid.

In the experiment comparing the effects of oral and subcutaneous treatment with hydrocortisone on urinary radiocalcium, the rats received 50 μc. of Ca^{45}/100 gm. body weight subcutaneously 53 days before urine collections were begun. Five days before steroid treatment, half of the rats were placed on a calcium-free diet, and were kept on this diet throughout the experimental period of 8 days. All rats received 15 gm. of food during the period of urine collections.

In the experiment in which the effect of calcium restriction and hydrocortisone upon bone resorption was studied, the rats received 100 μc. of Ca^{45}/100 gm. body weight, 53

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[2] Ca^{45} was used as the calcium chloride salt received on allocation from the Atomic Energy Commission.

TABLE 1. THE EFFECT OF PRETREATMENT WITH HYDROCORTISONE AND FOOD RESTRICTION UPON THE EXCRETION OF A SINGLE DOSE OF RADIOCALCIUM

Treatment	Hydrocortisone	Pair-weighed controls	*Ad libitum* controls
	% of injected radiocalcium excreted		
Urine	3.6**	0.59	0.66
Feces and Gastrointestinal Tract	31.5	34.5	26.9

** Significantly different from both groups of controls ($P < .01$).

days before collecting urines. Eleven days before steroid treatment, the stock diet was replaced by a calcium free diet (12). In all, the rats were maintained on this calcium-free diet for 23 days, the last twelve days being the urinary collection period. The period of pretreatment on a calcium free diet was twice as long as in the previous experiment.

In the experiment in which the effects of hydrocortisone on intact and nephrectomized rats were compared, the treated animals received 1 mg. of hydrocortisone/100 gm. body weight subcutaneously daily for three weeks. Bilateral nephrectomies were carried out via the abdominal route. Twenty-four hours before being sacirficed, each rat received 3 μc. of Ca^{45}/100 gm. body weight.

RESULTS

The effect of pretreatment with hydrocortisone on the absorption and excretion of an oral dose of radioacalcium

It can be seen from the data (Table 1) that the urinary excretion of radiocalcium by the hydrocortisone treated rats is about 5 times greater than that of either group of control rats ($P < .01$). There was no significant difference in combined intestinal content and fecal excretion of radiocalcium between the three groups.

Comparison between the effects of a single oral and intraperitoneal injection of hydrocortisone and prednisolone on urinary radiocalcium forty-eight hours after the administration of Ca^{45}

As can be seen from Table 2, the administration of both steroids resulted in an increase in radiocalcium excretion above the control values during the first 8 hours, especially after oral administration as compared to intra-

TABLE 2. THE EFFECTS OF AN ORAL AND INTRAPERITONEAL INJECTION OF HYDROCORTISONE AND PREDNISOLONE ON URINARY RADIOCALCIUM

Treatment	No. of animals	I.P.	Oral	I.P.	Oral
		Hours			
		0–8	0–8	8–24	8–24
Controls*	6	100	100	100	100
Hydrocortisone—2 mg./100 gm.	6	137	253**	89	95
Prednisolone—0.5 mg./100 gm.	6	136	230**	91	103

Radiocalcium was given 48 hours before urine collections were begun.
* The control count values were arbitrarily set at 100 for ease in comparison of the data.
** Significantly different from the control values ($P < .01$).

peritoneal injection. However, the excretion of Ca^{45} was comparable to the controls during the 8- to 24-hour period. Although prednisolone was given in 1/4 the dosage of hydrocortisone, it evoked about the same response.

The effect of hydrocortisone on urinary Ca^{45} and Ca^{40} of rats which had received Ca^{45} fifty-three days before steroid treatment

Singer and Armstrong have shown that about 53 days after the administration of radiocalcium to rats, the urinary excretion of the isotope represents a small uniform fraction of the administered dose (14). Any increase in urinary radiocalcium above the control values must be derived from the labeled calcium in bone, since this site is the body's principal reservoir of calcium.

TABLE 3. THE EFFECT OF HYDROCORTISONE ON THE URINARY EXCRETION OF CA^{45} AND C^{40} OF RATS KEPT ON STOCK OR CALCIUM-FREE DIET, FIFTY-THREE DAYS AFTER THE ADMINISTRATION OF THE ISOTOPE

	Stock diet				Calcium-free diet			
Weight change	Control −20 gm.	Hydrocortisone (s.c.) −79 gm.		Hydrocortisone (oral) −31 gm.	Control −48 gm.	Hydrocortisone (s.c.) −99 gm.		Hydrocortisone (oral) −79 gm.
No. of Rats	4	4		4	4	4		4
		Total counts excreted	*P values*			*Total counts excreted*	*P values*	
Urinary Ca^{45}								
1 & 2 days	37,000	18,000	<.05	42,000	24,000	26,000	N.S.	27,000
3 & 4 days	59,000	24,000	<.01	44,000	33,000	24,000	N.S.	25,000
5 & 6 days	79,000	92,000	N.S.	73,000	43,000	134,000	<.05	36,000
7 & 8 days	52,000	75,000	N.S.	46,000	38,000	145,000	<.01	34,000
Sum 0–8	227,000	209,000		205,000	138,000	329,000	<.05	122,000
Urinary Ca^{40}	*mg.*	*mg.*	*P values*	*mg.*	*mg.*	*mg.*	*P values*	*mg.*
1 & 2 days	4.6	5.5	N.S.	5.1	3.0	3.9	N.S.	3.4
3 & 4 days	5.0	6.1	N.S.	5.5	3.1	3.6	N.S.	3.2
5 & 6 days	7.2	10.4	<.05	6.3	4.4	6.3	<.05	4.9
7 & 8 days	4.9	8.9	<.05	4.9	3.8	6.5	<.01	4.2
Sum 0–8	21.7	30.9	<.01	21.8	14.3	20.3	<.05	15.2

The rats received Ca^{45} 53 days before treatment was begun.

After subcutaneous administration of hydrocortisone (Table 3), the usual delay or depression of urinary radiocalcium was observed for the first four days (11). This depression of urinary radiocalcium was significant in the rats maintained on a stock diet ($P < .05$ and $P < .01$) whereas, in the rats maintained on a calcium-free diet, no differences were observed. Beginning at day 5, and continuing through day 8, a significant increase in total calcium^{40} was observed in all rats which received the steroid subcutaneously. A statistically significant increase in urinary Ca^{45} was observed only in those rats which were kept on a calcium-free diet. Those on stock diet tended to have increased urinary Ca^{45} but the results were not significant.

The oral administration of hydrocortisone to rats on either stock or calcium-free diet, had no significant effect upon Ca^{45} or total calcium excretion.

The greater effectiveness of the subcutaneous route as compared to the oral is also evident by the greater weight loss, despite the fact that all animals consumed the same amount of food.

The effect of calcium restriction and hydrocortisone upon bone Ca^{45}

Here too, the isotope was administered 53 days before the steroid treatment was inaugurated. Thus, any increase in urinary radiocalcium again must be derived from bone.

There was no increase in urinary radiocalcium until day 5. From day 5 through day 12, there was a significant increase in urinary radiocalcium ($P<.01$) (Table 4).

TABLE 4. THE EFFECT OF HYDROCORTISONE UPON URINARY EXCRETION OF Ca^{40} AND Ca^{45} OF RATS KEPT ON A CALCIUM-FREE DIET

Body weight change, Gm.	Controls +40	Hydrocortisone 2 mg./100 gm. −50	P Values
	Counts excreted		
Urinary Ca^{45}			
1 through 4 days	35,000	34,000	N.S.
5 through 8 days	27,000	47,000	<.01
9 through 12 days	27,000	63,000	<.01
Sum 0–12	89,000	144,000	<.01
Urinary Ca^{40}, mg.			
1 through 4 days	5.5	4.7	
5 through 8 days	5.0	5.0	
9 through 12 days	5.1	5.4	
Sum 0–12	15.6	15.1	

The rats received Ca^{45} 53 days before urine collections.

In this experiment the rats received the calcium-free diet for 11 days rather than 5 days before urine collections were started. As a result of this longer pretreatment with a calcium-free diet, there was no difference in the total calcium excretion between the steroid treated rats and their controls despite the disparity in weight change.

Comparative effects of food restriction and hydrocortisone treatment upon the uptake and excretion of radiocalcium in normal and nephrectomized rats

The serum radioactivities, urinary radioactivities, and femur specific activities of the hydrocortisone treated intact rats were significantly different ($P<.01$) from those of either group of controls (Table 5). There was no difference between the pair-weighed controls and *ad libitum* feeding controls in the excretion of radioactive calcium, despite a difference in weight gain of 57 gm.

There were significant differences in serum radioactivities and femur specific activities between the nephrectomized *ad libitum* feeding controls and the nephrectomized hydrocortisone treated rats. These differences were of the same order as those seen in the intact rats. The serum activities of the nephrectomized pair-weighed controls were no different from those of the nephrectomized steroid treated rats. However, there were differences in the specific activities of the femurs ($P < .05$).

DISCUSSION

If hydrocortisone interferes with the intestinal absorption of calcium in the rat, one would expect to find after a single oral dose of Ca^{45}, more Ca^{45} in the gastrointestinal tract and feces and less in the urines of the steroid treated rats than in the control animals. However, this is not the case (Table 1) where about 5 times more isotope is found in the urines of the hydrocortisone treated rats, and no increase is seen in the gastrointestinal tract and feces. It appears that adequate absorption of calcium takes place but that there is an inhibition of the utilization of Ca^{45} which is reflected by the greater amount of isotope appearing in the 24-hour urines of the treated rats.

TABLE 5. THE EFFECT OF HYDROCORTISONE ON THE UPTAKE OF RADIOCALCIUM BY THE FEMURS OF INTACT AND NEPHRECTOMIZED RATS

Treatment	No. of rats	Body wt. change, gm.	Serum activity, c./min./ml.	Urine activity, c/24 hr. sample	Bone specific c./min./mg. Ca
Intact animals					
Hydrocortisone 1 mg./100 gm.	8	+ 24	7860	1.53×10^5	1160
Pair-weighed controls	8	+ 30	5630**	0.51×10^5**	1560**
Ad libitum feeding controls	8	+ 87**	6105**	0.47×10^5**	1720**
Nephrectomized animals					
Hydrocortisone 1 mg./100 gm.	8	+ 31	7295	—	1483
Pair-weighed controls	8	+ 30	7085	—	1843*
Ad libitum feeding controls	8	+110**	5590**	—	2110**

The rats received Ca^{45} twenty-four hours before being sacrificed.
* Significantly different from hydrocortisone treatment ($P < .05$).
** Significantly different from hydrocortisone treatment ($P < .01$).

It has been reported that the administration of the adrenal cortical steroids to rats invariably caused an increase in urinary radiocalcium, when the isotope was administered shortly before the steroid (11). Forty-eight hours after the injection of Ca^{45}, the level of circulating isotope was high and the isotope was still being incorporated into bone (14). If it is prevented from doing so, it will be excreted and presumably this is what occurred here (Table 2). Further evidence for the decreased utilization of Ca^{45} under the influence of hydrocortisone may be found in the experiment using nephrectomized rats (Table 5). Although the serum radioactivity levels are significantly higher in the hydrocortisone treated rats, (both intact and nephrectomized) the specific activities of the bones are significantly less, indicating less incorporation of isotope. Moreover, the steroid treated rats excreted much more Ca^{45} in their urine. In the intact rat, the possibility does exist that the decreased uptake of Ca^{45} by bone may be the result of the increased urinary Ca^{45}, however, this is not possible in the rats which had their kidneys removed.

Prednisolone, although administered at one quarter the dose level of hydrocortisone, has an equal effect upon urinary radiocalcium (Table 2). Clinically, prednisolone is about 4 times more active than hydrocortisone (15). Presumably, the more potent is the steroid in inhibiting bone formation, the greater will be its influence in preventing calcium utilization.

It has been shown that the duration of adrenal cortical steroid action depends on the route of administration (18). After daily subcutaneous injections, there was a slow continued release of the relatively insoluble steroids enabling them to continue their metabolic effects throughout the experimental period. However, after oral or intraperitoneal administration, there is a rapid disappearance of the steroid and its effects. Thus, the increased urinary Ca^{45} observed after oral or intraperitoneal injections at 8 hours is no longer evident at the end of 24 hours (Table 2). Even after 8 days of oral administration of hydrocortisone, there is no observable effect on radiocalcium excretion, indicating the rapidity with which the activity of the steroid disappears in the rat (Table 3). However, a significant increase is seen after subcutaneous administration (Table 3). This increase in urinary radiocalcium can only come from the bone since the animals received the isotope 53 days before. Although there appears to be an increase in Ca^{40} excretion on a calcium-free diet after subcutaneous administration of the steroid (Table 3), this does not occur if the animals are kept for a longer period of pretreatment on a calcium-free diet (Table 4). In this experiment, the rats were kept on a calcium-free diet for 11 days before being treated. Even after 12 days treatment with hydrocortisone at a level of 2 mg./100 gm. body weight subcutaneously, there is no increase in Ca^{40} excretion. Irving (19), has pointed out the importance of careful dietary preconditioning before patients can be used for calcium excretion studies. However, there is a striking increase in Ca^{45} excretion beginning at day 5 and continuing through day 8. This 4-day delay in the increased urinary Ca^{45} may be explained by assuming this is the time necessary for the normal resorptive processes in the bones to reach the site of the deeper areas of calcification which were formed 53 days earlier (20). The increased urinary radiocalcum must be derived from the Ca^{45} liberated by the normal resorptive or remodelling processes. However, under the influence of the steroid it is not re-utilized as efficiently and hence is excreted in the urine. If bone destruction had occurred, one would expect an increase in the total Ca^{40} in the urines of the treated animals. Since there is no detectable increase in total urinary calcium over that of the controls, the possibility that hydrocortisone mobilizes calcium from bone seems unlikely.

Assuming the calcium content of the skeleton to be 1% of the body weight, a 300 gm. rat would contain about 3 gm. of calcium. Taking the usual figure of about 30% skeletal calcium loss before osteoporosis can be recognized in man, a loss of 900 mg. would be required before osteoporosis would become manifest in the rat. Since the hydrocortisone treated rats lose only about 10 mg. Ca^{40} more than the controls in 8 days (Table 3), and approximately the same amount more via the feces, (unpublished) this would require about 360 days for these rats treated with 2 mg. of hydrocortisone daily to lose 900 mg. of calcium. It is possible that this may be part of the reason why the typical pathologic picture of osteoporosis has not been observed in the rat (16, 21) after steroid treatment, since to the best of our knowledge, rats have not been kept on the adrenal steroids for this length of time.

The exact mechanism whereby the adrenal cortical hormones inhibit calcium from being used for new bone synthesis is at present unknown. Albright (2) has hypothesized that the primary effect of adrenal cortical action on bone is to inhibit the synthesis of bone protein. It has been shown that the steroids do interfere with the synthesis of new protein (17). Perhaps the limiting factor may be in the synthesis of chondroitin sulfate which is not made as readily when steroids are administered (22). The primary site of adrenal cortical steroid action on bone still remains to be elucidated.

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QUANTITATIVE *IN VITRO* RESPONSE OF THYROID TISSUE TO THYROTROPIC HORMONE[1]

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ABSTRACT

In vitro uptake of iodine by beef thyroid minces was measured and found to be proportional to thyrotropic hormone content of the incubation medium. Such a response to TSH was not elicited from thyroid tissue in the form of homogenates. After two hours incubation in 4.0 ml. of Krebs-Ringer-phosphate buffer containing 10 to 20 μc. NaI^{131}, 800 mg. of minced beef thyroid displayed a mean iodine uptake of 22.6% (12.0 to 35.9) in the absence of added thyrotropic hormone. In medium containing 10 m.u./ml. of thyrotropin, mean uptake was 40.2% (22.0 to 69.3). When thyrotropic hormone concentration expressed in logarithms was plotted against % uptake there was a rectilinear response between 1 and approximately 10 m.u./ml. of TSH. The regression coefficient, determined by the responsiveness of the gland, was different for each mince. Analysis of variance of the regression lines indicated a very significant relation between dose and response. In this incubation medium iodine uptake was depressed by 6×10^{-4} M KI. Thyroxine at 4×10^{-5} M influenced neither initial iodine uptake nor response to thyrotropic hormone. Uniformity of response of thyroid minces and the magnitude of alterations in iodine uptake induced by TSH suggest the applicability of these techniques for *in vitro* estimation of thyrotropic hormone.

THYROID tissue has the unique ability to concentrate iodide converting it into iodine-containing organic compounds. The chemical reactions associated with such changes are dependent on several factors. Some are humoral and others are confined to the thyroid itself. A normally functioning gland is stimulated by thyrotropic hormone to take up iodide and to discharge the iodine-containing hormones into circulation. Chemical changes taking place during synthesis of these hormones have been investigated with I^{131} labeled iodide under both *in vivo* and *in vitro* conditions. A comparison of the compounds formed indicates that the reactions occurring in thyroid tissue are determined by viability, and in the case of thyroids studied under *in vitro* conditions by the degree of subdivision of tissue.

Although there is a difference in the compounds formed, it has been found that under both *in vivo* and *in vitro* conditions thyrotropic hormone

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increases utilization of I^{131}. Botkin *et al.* (1) examined thyroid glands removed from rats after administration of NaI^{131} and reported that the radioiodine content was related to previous exposure to thyrotropic hormone. Roche *et al.* (2) demonstrated that thyroid glands of rats cultured *in vitro* were stimulated by the anterior pituitary to synthesize iodothyronines. Effects of thyrotropic hormone on collection and release of iodide from beef thyroid glands were studied by Bakke and Lawrence (3). Bottari (4) reported that portions of thyroid incubated *in vitro* with thyrotropic hormone exhibited a degree of utilization of I^{131} proportional to the concentration of tropic hormone.

A single gland is not necessarily homogenous with respect to distribution of iodine-concentrating ability. A non-uniform binding of iodine in the whole gland has been indicated by Joftes and Warren (5) who found that in hamster thyroid more radioiodine was concentrated in the central follicles than those on the periphery. For accurate *in vitro* measurement of the response of thyroid tissue to thyrotropic hormone it was desirable to reduce tissue variability to a minimum. Therefore, in the study presented here several glands were employed and they were minced together to further reduce variability. The minces were incubated with I^{131} in media supplemented with various amounts of thyrotropic hormone and uptake of radioiodine was measured.

METHODS

Beef thyroid glands removed from animals within 30 minutes after death were brought to the laboratory in ice cold buffer. The buffer used in all experiments was Krebs-Ringer-phosphate pH 7.4 in which the .11 M $CaCl_2$ solution was replaced by an equal volume of 0.9% NaCl. For all manipulations ice cold solutions were employed. After the glands were freed of extraneous tissue they were minced with scissors and suspended in buffer. Following this washing, 800 mg. portions of the minced thyroid were weighed into 25 ml. Erlenmeyer flasks. To these were added buffer containing thyrotropic hormone at the level of 0 to 100 m.u.[2]/ml., 10 to 20 μc. of NaI^{131} and any other reagent which was to be examined for its influence on I^{131} uptake. The final volume was adjusted to 4.0 ml. with buffer. These flasks were incubated in the Dubnoff shaker at 37° C for two hours in an atmosphere of 95% O_2–5% CO_2. At the end of this period the tissue was immediately processed for measurement of radioactivity. The incubation mixture was transferred to a test tube with care to remove all tissue. The suspension was centrifuged for 20 min. at 2950 r.p.m. and the supernatant decanted. Tissue was resuspended in 5 ml. of buffer and centrifuged as previously. The washing was repeated twice. These manipulations were conducted at room temperature and required 2½ to 3 hours. Radioactivity was determined utilizing a probe type scintillation counter at a fixed distance from tissue. A solution of I^{131} equal to that added to the incubation medium was also counted and from these values the % uptake of iodine was calculated.

RESULTS

Increased amounts of radioactivity were taken up by thyroid minces fol-

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[2] m.u.—An abbreviation for milliunit, one-thousandth of a U.S.P. unit of commercially available thyrotropic hormone.

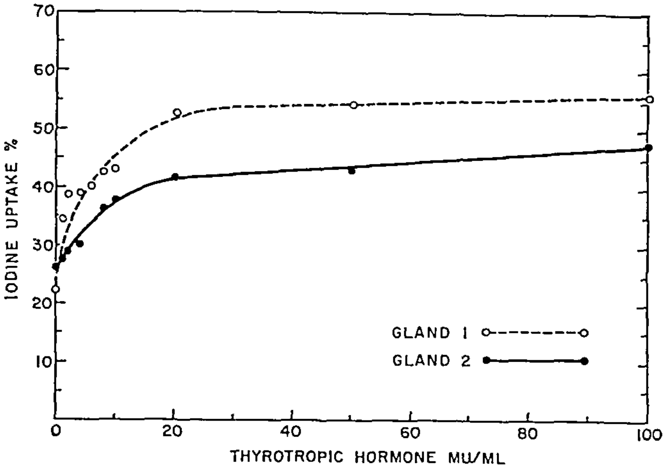

FIG. 1. *In vitro* I^{131} uptake of beef thyroid minces incubated in 0–100 m.u./ml. of thyrotropic hormone (TSH expressed as m.u./ml.)

lowing their incubation with TSH and I^{131}. The degree of uptake elevation was determined by the thyrotropic hormone content of the medium. Responses of typical thyroid minces to TSH at levels of 0 to 100 m.u./ml. are illustrated in Figure 1. Here it can be seen that iodine uptake increased in the presence of the tropic hormone until tissue radioactivity approached a maximum. This relation between dose and response may be shown as a sigmoid curve if % uptake is plotted against TSH expressed in logarithms (Fig. 2). From inspection of each curve the linear portion can be determined. In over 80% of a group of 60 minces response was linear from 1 to 8 or 10 m.u./ml. of TSH (Fig. 3). These are typical lines and show the effect on different thyroid minces of increasing concentrations of TSH. Regression coefficients of the minces which were studied ranged from 5.6 to 33.7. Application of the *t* test to iodine uptake at 0 and 1 m.u./ml. indi-

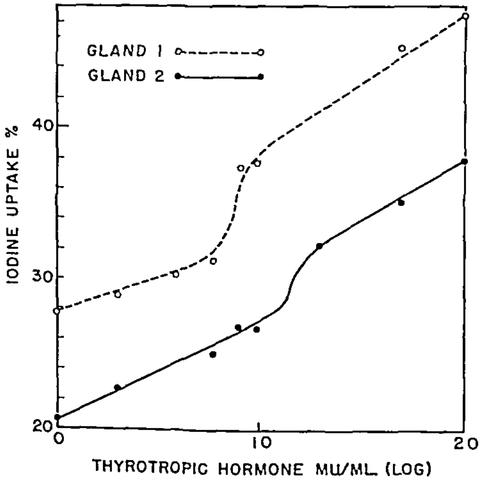

FIG. 2. *In vitro* I^{131} uptake of beef thyroid minces incubated in 0–100 m.u./ml. of thyrotropic hormone (TSH expressed as logarithm of m.u./ml.)

cated that it was possible to detect thyrotropic hormone at the level of 1 m.u./ml. ($P < .001$ to .005) in the incubation medium.

Average uptakes of radioiodine of a group of 60 minces are summarized in Table 1. Iodine uptake rose from 22.6 ± 5.3% in the absence of added hormone to 47.4 ± 7.8% when the medium contained 100 m.u./ml. of tropic hormone. Composite uptake data are plotted in Figure 4. There was a linear relation of dose and response between 1 and 8 m.u./ml. of thyrotropic hormone. An analysis of variance of the line indicated a P of .008 for the line, a figure which resulted from the variations in different glands. Such tissue differences were lacking when a single mince was employed. Homogeneity of a mince was reflected in a comparison of standard deviations in uptake of many minces with the deviation of duplicate samples of the same mince. Two to six samples of a single mince containing equal concentrations of tropic hormone were employed over the range 0 to 100 m.u./ml. and the standard deviation in uptake at every level of TSH for each mince was calculated. The average deviation of 20 such sets of figures at each tropic hormone level is found in Table 2. Also included are the

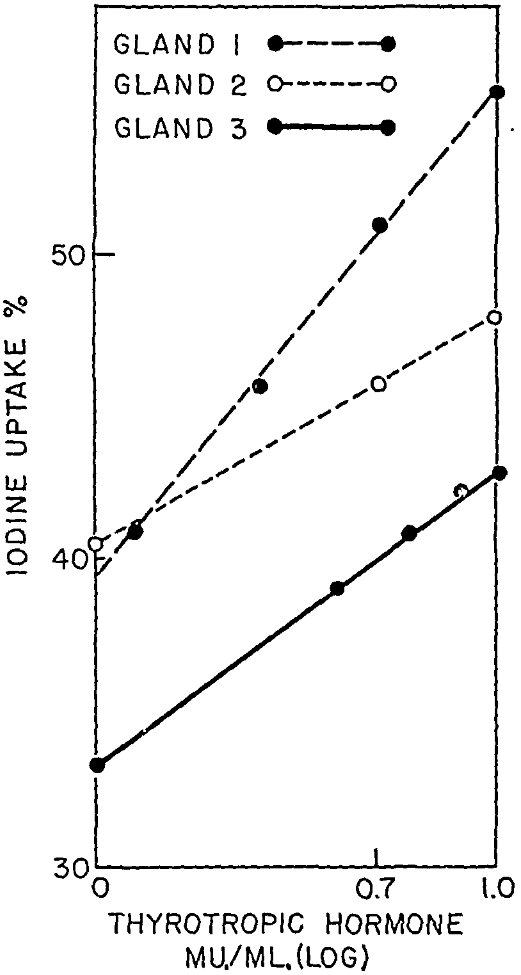

FIG. 3. *In vitro* I^{131} uptake of three different thyroid minces incubated in 0–10 m.u./ml. of thyrotropic hormone (TSH expressed as logarithm of m.u./ml.)

Table 1. Effect of thyrotropic hormone on iodine uptake of thyroid minces

TSH conc. m.u./ml.	% uptake I^{131}	
	Range	Mean ± std. dev.
0	12.0–35.9	22.6 ± 5.33
1	11.7–33.4	24.6 ± 5.18
2	14.8–39.3	27.3 ± 6.30
4	19.6–49.9	31.1 ± 7.99
6	20.7–56.7	35.3 ± 9.00
8	22.6–52.4	37.1 ± 8.38
10	22.0–69.3	40.2 ± 12.09
20	25.8–69.0	43.3 ± 11.82
50	33.9–68.0	46.0 ± 8.81
100	38.0–67.2	47.4 ± 7.82

standard deviations which resulted when uptake of 60 different minces were compared. The latter figures are five to six times as great as deviations of individual minces. Uniformity in uptake of duplicate samples gave indication of homogeneity of a mince. Because a single mince gave uniform response and each mince differed from the next with respect to both initial iodine uptake and response to thyrotropic hormone, a separate regression line was applied to each mince. An analysis of variance of such lines has indicated a $P < .001$ for the regression line, a figure which indicates a significant dose-response relation.

To account for the entry of I^{131} measured ultimately in thyroid minces, determination was made of the total uptake as opposed to the I^{131} which entered tissue during the washing period. For this study thyroid minces were incubated in the presence of different concentrations of thyrotropic hormone but without I^{131}. The latter was added at the end of the incubation period and the tissue washed by the usual procedure. Aliquots of incubation fluid and the three wash solutions as well as the tissue itself were examined for radioactivity. As seen in Table 3, four such studies indicated

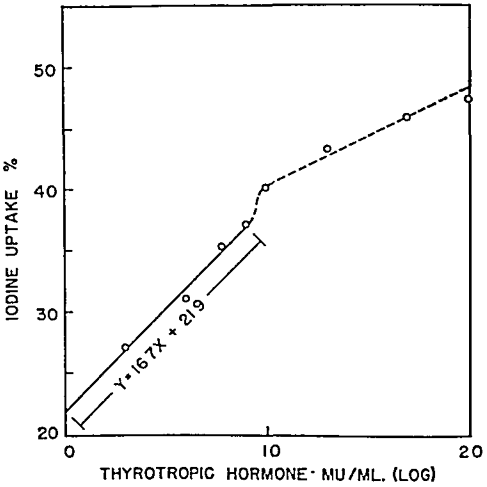

Fig. 4. Composite line representing *in vitro* iodine uptake of 60 different beef thyroid minces incubated in 0–100 mu./ml. of thyrotropic hormone (TSH expressed as logarithm of m.u./ml.)

TABLE 2. DEVIATIONS IN IODINE UPTAKE OF THYROID MINCES

TSH conc. m.u./ml.	% standard deviation	
	Pooled minces	Av. of 60 different minces
0	0.85	5.33
1	1.07	5.18
2	1.21	6.30
4	1.53	7.99
6	1.80	9.00
8	1.96	8.38
10	1.94	12.09
20	2.02	11.82
50	1.62	8.81
100	1.32	7.82

that with increasing concentrations of TSH slightly greater amounts of radioactivity entered tissue. Iodine taken up may be bound to protein or remain in the form of iodide. The trichloroacetic acid precipitable I^{131} in tissue and the combined incubation fluid and wash solutions was determined. Each 800 mg. of tissue was homogenized in buffer in an ice cooled Waring blendor. The suspension, 20 ml., was treated with 6.0 ml. of 40% trichloroacetic acid and the precipitate washed twice with 8.0 ml. of 5% trichloroacetic acid. The incubation fluid combined with solutions removed after washing the tissue (total volume approximately 20 ml.) was treated with trichloroacetic acid in the same manner. Radioactivity of the precipitates and aliquots of supernates was measured. As seen on Table 4 approximately 3% of the radioiodide added at the beginning of the washing period was precipitated with trichloroacetic acid. This value represented protein bound iodine, as well as adsorbed iodide and indicated that condi-

TABLE 3. DISTRIBUTION OF I^{131} ADDED TO THYROID TISSUE *in vitro*

Conc. TSH m.u./ml.	Fraction	Time of I^{131} addition	
		At beginning of 2 hr. incubation	At end of incubation
0	Tissue	26.8±2.3	8.7±1.4
	Supernatant	35.7±1.7	64.6±1.7
	1st Wash	14.5±2.2	13.7±3.8
	2nd Wash	11.6±0.3	7.1±0.1
	3rd Wash	8.9±3.1	4.7±1.0
10	Tissue	60.3±1.6	13.5±3.6
	Supernatant	16.3±0.8	61.8±4.8
	1st Wash	7.5±1.7	9.3±1.6
	2nd Wash	7.1±0.3	7.3±0.1
	3rd Wash	6.9±1.2	5.5±1.6
20	Tissue	69.0±1.7	14.9±2.5
	Supernatant	12.9±1.3	60.5±3.1
	1st Wash	5.0±1.3	9.1±1.3
	2nd Wash	5.4±0.4	7.3±0.4
	3rd Wash	5.8±1.5	5.7±1.5

These figures represent % of added radioiodide and are the average of four experiments.

tions which existed during the washing period were not conducive to conversions of large quantities of iodide to organic molecules.

The nature of radioiodine in tissue after incubation under the usual conditions i.e. in the presence of NaI^{131} and different concentrations of thyrotropin has been determined by applying similar techniques of trichloroacetic acid precipitation to the tissue as well as to the combined incubation and wash solutions. Data in Table 4 indicate that during incubation radioiodine precipitated with trichloroacetic acid was increased in the presence of thyrotropic hormone. If one compares the trichloroacetic acid precipitable I^{131} in tissue (Table 4) with the total radioiodine content (Table 3), approximately 50% (13.8/26.8) was protein bound in the absence of added thyrotropin. Increase of this fraction to 80% (48.3/60.3) was found when the preparation was supplemented with 10 m.u./ml. of TSH and to 84% (57.8/69.0) when 20 m.u./ml. were added. These figures agree with the well established relation of thyrotropic hormone to iodine uptake and utilization.

TABLE 4. NATURE OF IODINE COMPOUNDS PRODUCED AFTER *in vitro* INCUBATION OF THYROID TISSUE

Time of addition of I^{131}	Conc. TSH m.u./ml.	Fraction	Behavior in TCA	
			Precipitated	Soluble
Beginning of Incubation	0	Tissue	13.8	13.3
		Supernate	2.6	67.0
		Total	16.4	80.3
	10	Tissue	48.3	13.1
		Supernate	3.8	33.2
		Total	52.1	46.3
	20	Tissue	57.8	12.6
		Supernate	4.5	22.6
		Total	62.3	35.2
End of Incubation	0	Tissue	1.7	6.8
		Supernate	0.5	88.1
		Total	2.2	94.9
	10	Tissue	2.5	10.9
		Supernate	0.5	78.2
		Total	3.0	89.1
	20	Tissue	2.4	12.1
		Supernate	0.6	79.3
		Total	3.0	91.4

Figures represent % added radioiodide and are the average of 4 experiments.

The *in vitro* iodine uptake of an isolated thyroid gland was determined in part by the degree of subdivision of tissue. Uptakes of iodine by slices, minces and homogenates were compared. In this experiment a lobe of the thyroid was divided into equal parts. Frozen and fresh slices were prepared, the former with a freezing microtome set to give slices of 250 μ. Fresh slices 0.5 mm. thick were prepared using the Stadie-Riggs microtome.

TABLE 5. COMPARISON OF IODINE UPTAKE BY THYROID MINCES, SLICES AND HOMOGENATES

Samples/ Datum	TSH m.u./ml.	% uptake ± standard deviation			
		Form of tissue			
		Mince	Frozen slice	Fresh slice	Homogenate
5	0	18.4 ± 1.0	8.3 ± 2.4		
	10	35.2 ± 6.6	8.9 ± 1.3		
4	0	18.2 ± 1.1		13.9 ± 1.0	4.23 ± 0.9
	10	32.7 ± 2.5		32.2 ± 6.6	4.40 ± 0.6
5	0	30.6 ± 2.5	12.1 ± 0.7	21.4 ± 5.2	5.90 ± 0.2
	10	49.2 ± 2.7	12.1 ± 0.6	44.7 ± 10.3	5.97 ± 0.5

Eight hundred mg. of slices were incubated in each flask. Another portion of the gland was homogenized using 400 mg. of tissue per ml. of buffer. This was accomplished using an ice-cooled Waring blendor operated for 5 minutes. For incubation, 2 ml. aliquots were employed. As control, an equal portion of the same gland was minced by the usual technique. Radioactive iodine as well as thyrotropic hormone at levels of 0 and 10 m.u./ml. were added and the volume adjusted to 4.0 ml. with buffer. After incubation the homogenate was precipitated with 1.5 ml. of 40% trichloroacetic acid and the precipitate washed twice with 5 ml. of 5% trichloroacetic acid. These data (Table 5) indicate that I^{131} was taken up by thyroid tissue in all forms and that thyrotropic hormone increased uptake in fresh slices and minces but not frozen slices or homogenates. When tissue was in the latter forms the quantity of I^{131} concentrated was less than in fresh slices or minces.

TABLE 6. EFFECT OF VARIOUS REAGENTS ON *in vitro* I^{131} UPTAKE BY THYROID MINCES

Reagent Tested	TSH conc. m.u./ml.		
	0 % response*	10 % response	20 % response
5×10^{-4} M FMN	108.0 ± 9.0	85.4 ± 6.2	90.7 ± 6.3
5×10^{-4} M DPN	79.6 ± 10.0	72.5 ± 7.3	77.6 ± 3.8
1×10^{-3} M Ascorbic Acid	81.0 ± 5.8	74.4 ± 11.1	78.0 ± 9.2
1×10^{-3} M 8-Hydroxyquinoline	84.2 ± 11.5	56.7 ± 7.7	57.9 ± 2.6
4×10^{-7} M Thyroxine	101.3 ± 7.3	93.1 ± 5.7	93.1 ± 8.0
4×10^{-5} M Thyroxine	101.8 ± 6.7	93.4 ± 5.7	93.5 ± 4.3
6×10^{-6} M KI	98.4 ± 13.7	90.1 ± 5.9	92.0 ± 5.4
6×10^{-4} M KI	10.1 ± 1.6	6.8 ± 2.2	6.1 ± 2.1
1×10^{-3} M $CuSO_4$	90.0 ± 28.6	71.8 ± 32.2	66.4 ± 23.3
1×10^{-3} M $ZnSO_4$	95.5 ± 18.7	88.6 ± 12.9	90.5 ± 9.3
1×10^{-3} M $FeSO_4$	95.3 ± 17.2	97.2 ± 12.9	90.6 ± 14.5
1×10^{-3} M $FeNH_4(SO_4)_2$	86.2 ± 14.6	96.2 ± 10.1	96.9 ± 3.4
1×10^{-3} M Na_2SO_4	99.7 ± 8.0	98.8 ± 7.9	99.3 ± 6.7

$$* \ \% \text{ Response} = \frac{\% \text{ iodine uptake in the presence of reagent tested}}{\% \text{ iodine uptake in unsupplemented buffer}}.$$

These values based on six experiments for each reagent.

TABLE 7. EFFECT OF INCUBATION CONDITIONS ON IODINE UPTAKE

TSH m.u./ml.	% uptake I^{131}					
	Conditions					
	37° C 95% O_2-5% CO_2	37° C N_2	37° C Air	4° C 95% O_2-5% CO_2	4° C N_2	4° C Air
0	15.66	10.10	14.54	12.50	7.49	12.24
10	33.84	13.43	34.24	16.26	6.78	12.05
20	42.18	9.00	38.78	17.54	8.16	13.25

Iodine uptake was measured in media containing agents affecting enzyme activity and thyroid function. Two reactions were considered, first iodine uptake itself as seen in media unsupplemented with TSH and second, response to thyrotropic hormone. As seen in Table 6, the magnitude of standard deviations limited evaluation of all but a few of the reagents studied. 8-Hydroxyquinoline had little effect on iodine uptake but decreased response to thyrotropic hormone. Both uptake and stimulation by thyrotropin were impaired by DPN, ascorbic acid and 6×10^{-4} M KI. Influence of the other reagents on both of these phenomena was questionable because of the variety of responses of different minces. These responses reflected differences in tissue and were due to the reagent in question since the reactions were unaffected in solutions of equal molarity containing Na_2SO_4.

Iodine uptake in the presence and absence of thyrotropin appeared to depend on temperature and gaseous atmosphere during incubation. As seen in Table 7 replacement of 95% O_2−5% CO_2 with air had little effect on iodine uptake or response to thyrotropic hormone. In an atmosphere of nitrogen, uptake was reduced and response to tropic hormone disappeared. For these tissue reactions 37° C was more favorable than 4° C.

Responses of an isolated thyroid mince differed according to the weight of tissue employed. Iodine uptake of different amounts of thyroid tissue is seen in Table 8. With quantities greater than 800 mg., uptake was not appreciably increased in 10 m.u./ml. of thyrotropin. Such limitation of response may be due to depletion of I^{131} by tissue. Theoretical aspects of

TABLE 8. *In vitro* UPTAKE OF I^{131} BY THYROID MINCES AND THEIR RESPONSE TO TSH-EFFECT OF TISSUE WEIGHT

TSH m.u./ml.	% uptake I^{131}					
	Tissue weight, mg.					
	200	400	800	1000	1500	1800
0	8.8	14.4	24.4	30.3	35.2	36.8
10	27.1	35.0	49.1	52.0	48.0	53.9

Incubated in 4.0 ml. buffer at pH 7.4, 2 hrs. 37° C in 95% O_2-5% CO_2.

radioiodine distribution between tissue and incubation medium have been discussed by Freinkel and Ingbar (6).

Rate of iodine incorporation was studied by measuring % uptake at 30 minute intervals over a 3 hour incubation period. The flasks contained 0, 10 and 20 m.u./ml. of thyrotropin. Uptake rate curves of one experiment are seen in Figure 5. Four such experiments had two results in common: 1. The most rapid reaction occurred during the first 30 minutes. After this, rate was lower and represented removal of a constant portion of the unreacted I^{131} with variation of approximately ±1% in the absence of thyrotropic hormone and ±10% in its presence. 2. The initial velocity increased with addition of thyrotropin. In one experiment, in 0, 10 and 20 m.u./ml. of TSH, velocities of 0–30 min. were .4007, .4680 and .4797%/minute respectively.

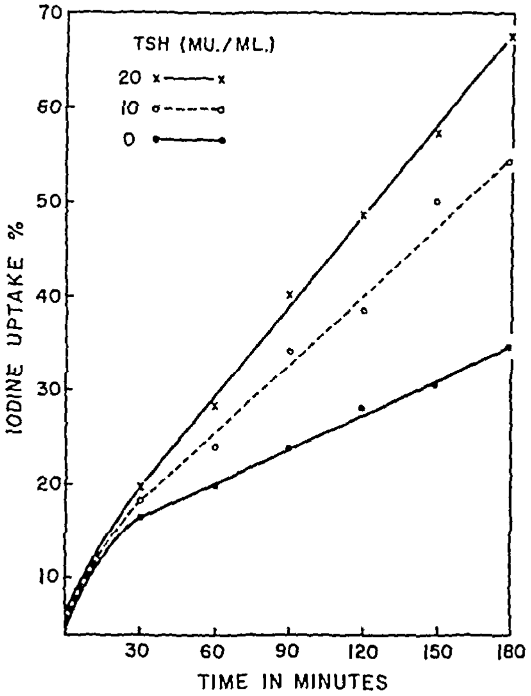

FIG. 5. Effect of thyrotropic hormone on rate of *in vitro* I^{131} uptake.

DISCUSSION

Iodine uptake by thyroid tissue requires several enzyme systems catalyzing a series of consecutive reactions. With establishment of a steady state all reactions proceeded at a rate which depended on concentration of enzymes, activators and inhibitors in tissue. Alterations in these factors determined ultimately the curve representing effect of thyrotropic hormone on uptake. At low hormone levels a linear relation was observed when radioiodine uptake was plotted against logarithm of thyrotropin concentration. As TSH content was increased, there was a tendency for the curve to become sigmoid.

Absolute differences in uptake of tissue after 2 hours incubation in thyrotropic hormone permitted detection of the effect of thyrotropin on accumulation of both free iodide and protein bound iodine. As indicated by solubility in trichloroacetic acid, TSH increased I^{131} taken up as iodide if the radioiodide were added at the end of the incubation. When tissue was in contact with I^{131} during the entire incubation period, such accumulation of iodide was not apparent due probably to the formation of PBI^{131}. Thyrotropic hormone increased protein bound iodine of both tissue and incubation fluid. The latter illustrated a well established phenomenon associated with thyrotropic hormone—promotion of the discharge of protein bound iodine from the thyroid.

The values for iodine uptake reported in this study may be compared with those of other investigators who measured *in vitro* iodide utilization. Iodide uptake and release was studied by Bakke and Lawrence (3) who observed that uptake was greater and release was less from thyroid tissue incubated in media supplemented with propylthiouracil and thyrotropic hormone. Radioactivity released was analogous to the I^{131} of the wash solutions shown on Table 3 for flasks to which radioiodine was added at the beginning of the incubation. Tropic hormone decreased the I^{131} which could be removed on washing. The influence of blocking agents on the nature of the iodine uptake has been studied by Freinkel and Ingbar (7) who showed that I^{131} concentrated by mammary slices from media supplemented with tapazole was entirely soluble in trichloroacetic acid. Chromatographically it was identified as inorganic I^{131}. Elimination of tapazole resulted in the formation of significant amounts of trichloroacetic acid insoluble material. Taurog *et al.* (8, 9) also demonstrated on chromatograms the formation of protein bound iodinated compounds in slices and particulate fractions of the thyroid. The so-called iodine uptake reported in the present investigation represented protein bound iodine as well as trapped iodide. Bakke and Lawrence (3) found that collection of iodide under *in vitro* conditions was dependent on medium volume and carrier iodide concentration. To allow for variations in medium volume and tissue weight Freinkel and Ingbar (7) expressed iodine uptake from a tapazole containing medium as the concentration gradient S/M

$$\left(\frac{\text{counts/gm. of tissue}}{\text{counts/ml. of medium}}\right).$$

An analysis of the data on Table 3 has been carried out along similar lines in which the I^{131} mince/medium ratio has been expressed. In 0, 10 and 20 m.u./ml. of thyrotropic hormone values of 1.8, 7.6 and 11.2 were found when I^{131} was added at the beginning of the incubation period. If radioiodine was added at the end of the incubation little trichloroacetic acid precipitable iodine resulted. The mince/medium ratios in the corresponding concentrations of thyrotropic hormone were 0.48, 0.78 and 0.87. These values are not precisely comparable to the data reported by Freinkel and

Ingbar since no tapazole was added to the medium. However, they indicate that the values reported in this study for uptake are accompanied by a low but genuine error due to the iodide trapped because of the effect of the tropic hormone on osmoregulation. This error might be eliminated by amplifying the procedure to include a separation of the trichloroacetic acid precipitable iodine in tissue and incubation fluid. The authors declined routine use of this modification because it lengthened the assay period by one day.

Although thyroid tissue displays an ability to concentrate iodine, the nature and rate of reactions taking place depend upon conditions employed for study. After culturing thyroid glands *in vitro* for 25 days, Roche *et al.* (2) noted that the synthetic mechanism was not modified *in vitro* although under these conditions hormone formation was less intense, and following stimulation with thyrotropic hormone both triiodothyronines were formed in preference to thyroxine. Morton and Chaikoff (10) reported that incubation of slices of thyroid glands with NaI^{131} was associated with the production of diiodotyrosine and thyroxine. These conversions were reduced if tissue was minced and lost if tissue were homogenized. Taurog *et al.* (8) found that in whole homogenates there was formation of monoiodotyrosine but no diiodotyrosine. In our experiments, the physical form of tissue limited the response to thyrotropic hormone and iodide uptake. Uptake occurred when thyroid tissue was in any state of subdivision but response to thyrotropic hormone was lost when tissue organization was disrupted by homogenization or freezing. Such responses of thyroid tissue suggest an association of these reactions with the distribution of I^{131}. Microscopic examination of the tissue employed indicated that the mince differed little from a section of an intact thyroid gland. Both showed regular thyroid follicles filled with colloid and lined by cuboidal epithelium. Thyroid homogenates showed an occasional isolated follicle against a background of amorphous fragmented eosinophilic material and debris. A frozen slice contained follicles characterized by the absence of colloid.

Sensitivity of thyroid tissue to thyrotropic hormone under *in vitro* conditions has been studied. Bakke and Lawrence (11) found that iodine collection of slices was stimulated by as little as 1×10^{-2} m.U. of TSH whereas 8 m.U. were required for organic binding. As reported by Lawrence and Bakke (12) water transfer of thyroid slices was altered by 8×10^{-5} U./ml. Using tissue culture techniques, Bottari (13) measured iodine uptake of portions of thyroid glands and detected 1×10^{-5} I.U. This figure converted to U.S.P. units with factors summarized by Overbeek *et al.* (14) is equivalent to .05 U.S.P. m.U. An *in vitro* technique was reported by Florsheim *et al.* (15) who found that incorporation of P^{32} in phospholipids of thyroid slices was regulated by TSH with a linear response between 2 and 200 m.U./ml. In our experiments, there was detection of at least 1 to 2 m.U./ml. of thyrotropic hormone. All glands were not equally responsive to TSH, and sensitivity was reflected in the slope of the regression line. The steeper curves relating response to dose provided a more precise estimate of different concentrations of thyrotropin and also permitted the detection of lower levels of TSH. In order to increase the accuracy of measuring thyrotropic hormone in this *in vitro* system, an arbitrary standard was employed in evaluating alterations in iodine uptake. A thyroid mince was expected to display a difference of 10% in uptake between 0 and 10 m.u./ml. of thyrotropic hormone. In a group of 68 minces, 60 met such a standard indicating that 88% of the minces displayed satisfactory response to TSH. Because of the uniformity of response of beef thyroid minces, it is suggested that this procedure may be applicable as an *in vitro* assay for thyrotropic hormone. Although not maximally sensitive, the technique is simple, rapid and accurate. It minimizes variability of thyroid tissue and is not affected by thyroxine.

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THE POSSIBLE ROLE OF OXYTOCIN IN STIMULATING THE RELEASE OF PROLACTIN[1]

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ABSTRACT

Hypothalamic lesions in the supraoptico-hypophysial tract of lactating rats produced a block of both milk ejection and milk secretion. The defect in milk secretion was correlated with the deficiency in endogenous oxytocin secretion, but there was no correlation between polydipsia and milk ejection or milk secretion. The effective lesions interrupted the supraoptico-hypophysial tract either in the median eminence or in front of this structure. The defect in milk secretion was seen in rats where median eminence, hypophysial portal vessels, and anterior lobe of the pituitary appeared to be essentially normal on histological examination. Pitocin and synthetic oxytocin delayed mammary involution in lactating rats. Pitressin produced a smaller effect. The effect of Pitocin and synthetic oxytocin persisted in rats with hypothalamic lesions. Since a defect in endogenous oxytocin release led to a defect in milk secretion, whereas oxytocin delayed mammary involution, it was postulated that oxytocin may be the neurohumor stimulating the release of lactogenic hormone.

PREVIOUS work by a number of investigators has indicated that vasopressin or a closely related polypeptide may play a role in the release of adrenocorticotrophin (ACTH) (1, 2, 3). In view of this relationship, it became of interest to speculate on a possible role of the other neural lobe hormone, oxytocin, in regulating anterior pituitary function. Andersson (4), and Cross and Harris (5) demonstrated that suckling produces a reflex release of oxytocin which in turn causes milk ejection. Selye (6) and others (7, 8) have further shown that suckling stimulates milk secretion by a reflex augmentation of the release of lactogenic hormone. The principal evidence for this view is the demonstration that mammary involution is delayed by the continued suckling of breasts from which the egress of milk has been prevented. Since lactogenic hormone has a similar effect in delaying mammary involution, Hooker and Williams suggested that suckling must stimulate a discharge of this hormone (9). Because suckling releases both oxytocin from the neurohypophysis and lactogenic hormone from the anterior lobe, it is reasonable to postulate that the release of oxytocin may stimulate the release of lactogenic hormone. This suggestion was apparently first made by Peterson (10). Subsequently, Benson and Folley reported that oxytocin delays the involution of the

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breast which follows weaning (11), which provided the first experimental evidence for the postulated role of oxytocin in the release of lactogenic hormone.

The present experiments were designed to test the hypothesis that oxytocin triggers the release of lactogenic hormone in the rat. In the first series of experiments, the effect of hypothalamic lesions designed to prevent the secretion of oxytocin was studied. Then, the effect of exogenous oxytocin on the course of mammary involution was determined in normal rats and those with hypothalamic lesions. A defect in milk secretion as well as milk ejection was demonstrated in the rats with lesions, whereas exogenous oxytocin delayed mammary involution in both normal rats and those with lesions. A preliminary report of this work has already appeared (12).

METHODS

Lactating Wistar rats were used throughout this study. They were fed a diet of Purina chow supplemented with oranges and bread.

Milk secretion was estimated in most cases simply by recording to the nearest 0.1 gm. the daily weight gain of the litter, and then converting this to a rate of gain by expressing it in mg. gained/gm. of weight of litter/hour. Using data of Brody (cf. 13) it is possible to estimate milk yield by multiplying gain of body weight by 100/55.

Where oxytocin was injected to cause milk ejection, a direct measure of milk yield was attempted by weighing the litter before and $\frac{1}{2}$ to 1 hour after oxytocin administration.

In rats with failure of oxytocin release where litters were suckled on alternate days by normal mothers, the calculation of total milk flow of deficient mothers was more complicated. Two different methods of calculation were used and compared—one based upon daily weight gain of the litter, the other upon gain of the litter following oxytocin injection into the deficient mother. These calculations are more fully described in the appropriate section of the results.

A. *Lesion experiments:* On the second day of lactation each litter was reduced to six members. If the litter was gaining weight hypothalamic lesions were produced in the mother on the fourth day of lactation, though in some instances they were made later, i.e., as late as the eighth day. Lesions were produced with the Krieg stereotaxic instrument with the rat under ether anesthesia as described previously (1). Coordinates were used which had been shown to produce lesions that interrupted the supraoptico-hypophysial tract either in the median eminence or in front of this structure. "Large" lesions were made by coagulating bilaterally with a current of 6 mA. for 15 seconds, whereas "small" lesions were made with a current of 2.4 mA. for 5 seconds. After a two-hour recovery period, the mothers were returned to their litters. In rats with median eminence lesions, where a defect in milk ejection was thought likely to occur, an injection of commercial oxytocin (Pitocin, 0.75 Units (U.) diluted to 0.7 ml. with physiological saline, intraperitoneally (I.P.)) was given several hours after the operation. Thereafter, whenever the litters nursing mothers with lesions lost weight overnight, these mothers were injected 1–3 times a day with oxytocin beginning that morning. If lactation was interrupted following placement of the lesions in spite of oxytocin injections, the litters of the operated mothers were exchanged with those of foster mothers every 24 hours. In this way continued suckling of the rats with lesions was assured, and litters could be kept in good condition, though somewhat subnormal in weight, throughout the experiment.

In order to provide control data for these experiments, normal mothers were isolated from their litters overnight and then anesthetized with Nembutal (4 mg./100 gm., I.P.). The milk obtained by the litters from the anesthetized rats before and after the injection of oxytocin was then determined by weighing the litter.

Water intake and body weight of the mother rats with lesions were recorded at frequent intervals. At autopsy, the breast tissue was examined grossly, and in some instances histologically after fixation in 10% formalin, paraffin imbedding, sectioning at 5μ, and staining with hematoxylin and eosin. The right ovary and left adrenal were dissected free of fat and weighed to the nearest 0.1 mg. on a torsion balance. Uteri were examined but were not weighed. Brains with pituitaries attached were preserved in either formalin or Bouin's solution. Diencephalon and pituitary were serially sectioned at 5 or 15μ, and every 5th or 10th section mounted. Diencephalons were stained with either Luxol fast blue (14) or the chrome-alum hematoxylin phloxine method of Gomori (15). Pituitaries were stained with either hematoxylin-eosin, azo-carmine, or chrome-alum hematoxylin phloxine.

B. *Injection experiments:* To study the effect of possible neurohumors upon lactation in mothers not permitted to suckle their young, litters were again reduced to 6 members on the 2nd day of lactation and removed on the 4th day. All mother rats were then injected, I.P., thrice daily for 9 days with 0.3 ml. of either physiological saline, Pitocin, Pitressin, or synthetic oxytocin (Syntocinon[2]). Hypothalamic lesions were made in certain rats as described for "small" lesions, immediately after removal of their litters. The remainder had no hypothalamic injury. At sacrifice, the left abdominal breast was examined grossly and histologically after preparation as described under A.

RESULTS

I. The Effect of Hypothalamic Lesions on Milk Ejection and Lactation in the Rat

A. *Normal Rats.* Five mother rats gave milk at such a rate that their litters gained an average of 3.7 mg./g. of litter/hr. during the first 2 weeks of lactation (Table 2). This rate of gain served as the control value for rats with lesions where a foster mother was not required. The foster mothers, which were used if lactation was interrupted after lesions, gave milk at a more rapid rate, providing an average weight gain by each litter of 5.6 ±0.4 mg./g./hr. This was probably due to the fact that under these conditions the mother was supplying most of the milk for 2 sets of litters of 6 infants each. The increased suckling stimuli apparently resulted in a greater milk yield by the mother. These mothers were considered the controls for rats with lesions whose litters required foster mothers.

Litters which were isolated from their mothers lost weight at a rate of 2.8 ±0.17 mg./g./hr. When placed with Nembutal-anesthetized mothers which had been isolated overnight to allow milk to accumulate, the litters were able to obtain no milk (Table 1), indicating that anesthesia completely blocks milk ejection and that no milk is obtained by passive withdrawal from breasts containing milk in the rat. This confirms the extensive observations of Grosvenor and Turner (16). Since no milk is obtained in the absence of oxytocin, weight change of a suckling litter in the absence of injected oxytocin has been considered an index of the degree of failure in endogenous oxytocin secretion in rats with lesions. Of course if no milk is present for ejection, the index is useless.

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[2] Syntocinon was kindly supplied by Dr. S. Gimpel of Sandoz Co., Hanover. New Jersey.

The injection of oxytocin in these anesthetized rats produced a rapid milk ejection and permitted the litters to obtain milk (Table 1). The maximum amount obtained under such conditions provides an estimate of the capacity of the litter to ingest milk after isolation and a single injection of oxytocin. This maximum value was obtained by isolating foster mothers overnight since they might be expected to accumulate more milk than normal mothers. It appears that the litter which has been fasted for a period of 15 hours can ingest at least 9–10% of its body weight in milk after a single injection of oxytocin has been given to the anesthetized foster mother. The mean litter weight gain in 5 such experiments was 9.2% of body weight. This measurement provides information concerning the maximum weight gain of litters which might be expected after an injection of oxytocin into a mother with a deficit of endogenous oxytocin release.

TABLE 1. THE EFFECT OF ANESTHESIA ON MILK EJECTION IN THE RAT

Weight gain of litter (grams) during 30 minute periods			
Rat No.	Isolated	Suckling a mother rat under nembutal	
		No oxytocin	Oxytocin*
1	−0.1	−0.1	1.6
2	−0.15	−0.1	2.5
3	0.0	−0.3	1.7
4	−0.2	−0.3	5.6
Mean	−0.1	−0.2	2.8

* 0.75 I.U. Pitocin given intraperitoneally.

B. *Rats with Lesions Which Did Not Block the Release of Oxytocin.* The results obtained in 14 rats where the lesion did not prevent the infants from obtaining milk are illustrated in Table 2. As indicated in the table, these lesions were in various hypothalamic areas. Most lesions appeared to produce a slight decrease in weight gain of litters when compared with litters from control, non-operated mothers. The mean weight gain of all rats was 9% less than that of control litters (Fig. 3). It was noted that most animals gave very little milk on the first post-lesion day, and this accounts for much of the decrease in weight gain of these litters. The remainder of the slight deficiency in these rats may be accounted for by the slight weight loss of the mothers, suggesting a decrease in food intake, and by the fact that, in some cases some damage was certainly sustained by the supraoptico-hypophysial tract. Such damage is shown in section C to produce an alteration in milk secretion. Because Olivecrona (17) has found that destruction of the paraventricular nuclei results in a decrease in neural lobe oxytocin content, lesions were placed in an attempt to destroy the paraventricular nuclei in 5 cases (Table 2, E, paraventricular region). These nuclei sustained severe damage in 4 rats with a cell loss estimated to be 50–75%; in the 5th rat (P-7), however, a nearly complete destruction of the paraventricular nuclei was achieved (estimated cell loss>95%). The observation that there was little or no impairment of lactation in these cases can be considered as evidence against an essential role of these nuclei in oxytocin release or lactation.

TABLE 2. RESULTS IN RATS WITH LESIONS WHICH FAILED TO BLOCK LACTATION

Rat No.	Mean litter weight gain (mg./g./hr.)	% of control[1]	Right ovarian wt. (mg.)	Left adrenal wt. (mg.)	% change in body weight of rat	H_2O intake (ml./day)	Size lesion
A. Preoptic Area							
12	2.7	85			−17	N[2]	L[3]
B. Dorsal Hypothalamus and Ventral Thalamus							
CL7	3.0	89			−16	16	S[3]
CL6	4.0	105			−15	22	S
CL5	2.2	77	17	19	− 8	28	S
1C	2.6	83	27	23	− 7	34	S
CL3	3.0	89	29	28	+ 9	50	S
Mean	3.0	89			− 7	30	
C. Lateral to Median Eminence							
4	3.0	89	32	39	− 9	34	S
6	2.8	86	50	63	− 9	45	S
D. Posterior to Median Eminence							
2	2.8	86	30	52	− 2	N	S
3	2.8	86	47	57	− 9	N	S
E. Paraventricular Region							
P-1	3.8	102	55	34	− 1	91	S
P-2	4.0	105	51	37	−14	48	S
P-3	2.5	82			− 2	40	S
P-4	3.7	100			−16	62	S
P-7	3.9	103	37	43	− 5	43	M[3]
Overall Mean:	3.1	91	38	39	− 8		
Mean of 5 controls	3.7	100					

[1] For method of calculation, see page 876.
[2] Approximately normal.
[3] L = "Large" lesions; M = "Medium" lesions; S = "Small" lesions.

Two of the rats with lesions in the paraventricular nuclei exhibited a mild polydipsia which is attributed to the fact that the lesions in both cases extended to the base of the brain so as to produce an injury to the supraoptico-hypophysial tract. In neither case was this injury sufficient to cause a block of milk ejection or secretion.

C. *Rats with Lesions Which Interfered with the Release of Oxytocin.* Lesions designed to interrupt the supraoptico-hypophysial tract, and thereby prevent the release of oxytocin, produced a different picture (Table 3A). In these cases the infants were able to obtain little or no milk despite vigorous suckling. They lost weight when left with the mother rat, which was taken to mean that the milk ejection reflex was interrupted by the deficiency of endogenous oxytocin secretion. These rats were enabled to obtain a certain amount of milk by periodic injections of oxytocin, thus confirming the earlier findings of Cross and Harris (5) in the rabbit and Benson and Cowie (18) in the rat.

TABLE 3A. LACTATIONAL PERFORMANCE OF RATS WITH LESIONS IN THE SUPRAOPTICO-HYPOPHYSIAL TRACT

Rat No.	Mean litter weight gain (mg./g./hr.)			% of control*	Right ovarian wt. (mg.)	Left adrenal wt. (mg.)	% change in body weight of rat	H_2O intake (ml./day)
	Without oxytocin	After oxytocin	Total					
1. "Large" Lesions								
4	−3.1	+0.6[1]	−2.5	4	37	25	− 7	200
1D	−3.1	+2.3[3]	− .8	24	40	35	−10	110
2D	−2.1	+1.7[3]	−0.4	29	45	40	−12	65
4D	−3.5	+1.3[3]	−2.2	7	34	27	0	35
Mean:	−3.0	+1.5	−1.5	16	39	32	− 7	102
2. "Small" Lesions								
2B	−3.3	+1.7[1]	−1.6	14	46	24	+13	24
5B	−3.1	+3.2[1]	+0.1	34	44	39	−12	150
6B	−3.5	+2.7[1&3]	−0.8	24	32	54	−16	85
7B	−4.3	+1.8[1&3]	−2.5	4	45	35	− 8	35
8B	−3.4	+2.1[1&3]	−1.3	18	47	48	−13	37
A5	−3.1	+2.2[3]	−0.9	23	37	45	−14	170
A4	−1.8	+1.8[3]	0.0	33	55	40	− 6	84
Mean:	−3.2	+2.2	−1.0	21	40	41	− 8	84
3. Partial Lesions								
9B	−2.1	+4.2[3]	+2.1	75	37	53	− 9	240
5	−1.9	+2.9[3]	+1.0	58	47	41	− 8	40

* For method of calculation see page 876.

[1] One injection of oxytocin per day.

[3] Three injections of oxytocin per day.

The marked reduction in milk secretion in these rats made it necessary to employ foster mothers for the infants. Litters were switched back and forth daily between the two mothers so that suckling stimuli were maintained at all times. The routine consisted of switching the litters one hour after the deficient mother received her morning oxytocin injection; thus, the hungry infants which had been unable to obtain milk overnight from the rat with lesions were enabled within this hour to obtain the milk that had accumulated since the last injection. Because the litter was switched to the foster mother after this large intake of milk, it was impossible to use the simple recording of litter weight as an index of lactation in the rats with these effective lesions. Instead, the sum of the weight gains during a 24 hour period, following the individual oxytocin injections, expressed as mg./g. of litter/hour was determined. Then, the weight change of the litter overnight in the absence of injected oxytocin was calculated as mg./g. of litter/hour. (This was taken as an index of the ability of the infants to obtain milk by secretion of endogenous oxytocin since it has been shown that rats do not obtain milk by passive withdrawal from the breast.) The sum of the weight gains occurring after oxytocin injections was added to that obtained in the absence of the drug to give the total weight gain of the litter. This was then compared with the average weight gain of each litter when nursing the control foster mother to determine the percentage deficit in lactation.[3] This method overestimates the lactational performance of the rats with lesions since all milk obtained after injection of oxytocin is not converted to tissue. In two cases where it was necessary to use oxytocin but a foster litter was not required (Table 3, Partial lesions), the actual weight gain of the litter was compared with the calculated gain using the above method. It was found in both instances that the calculated weight gain was greater than the actual gain; the cumulative error at the end of 11 days was 28 and 32%. In spite of this error it is felt that this method is a useful one and that it gives values which are closely related to lactational performance.

An alternative method of comparison of the lactational performance of rats with lesions with that of the foster mothers is to convert the weight gain of the litters nursing foster mothers into an estimate of milk yield. According to Brody (cited by Cowie and Folley (13)), the weight gain of a litter is roughly equivalent to 55% of the milk yield. Consequently an estimate of the milk yield can be made by multiplying the litter gain by 100/55. This value, which for the foster mothers is 10 mg./g./hr., can then be compared with the milk yield obtained after oxytocin in rats with effective lesions. A good estimate of the percentage reduction in milk yield in rats with lesions results since these rats give almost no milk during the periods when oxytocin is not given. Both methods of calculation of the lactational performance of the rats with lesions agree surprisingly well.

"Large" lesions in the supraoptico-hypophysial tract caused the litters to lose weight at a rate of 3.0 mg./g./hr. when oxytocin was not administered (Table 3A). Oxytocin injections permitted a weight gain at a rate of 1.5 mg./g./hr. Thus, in spite of injections of oxytocin which permitted removal of milk from the mothers, the litters lost weight at a rate of 1.5 mg./g./hr. When these litters were suckling foster mothers on alternate days, they gained weight at a rate of 5.6 mg./g./hr. Thus, in these rats there was a deficiency of both milk ejection (gain without oxytocin) and milk secretion (total weight gain). The latter was estimated to be only 16% of normal (Table 3A, Fig. 3). This marked reduction in milk secretion was reflected in a decreased size and partial involution of breast tissue when examined grossly and microscopically. The complete course of the experiment in one of these cases is shown in Figure 1. It is apparent that little milk was obtained in the absence of oxytocin and that, even when it was given, the milk yield was grossly subnormal. The greatest amount of milk was obtained on the first post-operative day. This was followed by a gradual decrease in milk yield to a minimum value on the 5th postoperative day. In most other cases the maximum milk yield also occurred in the immediate post-operative period.

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[3] The method of calculation was as follows: if when suckling the foster mother the litter gained weight at a rate, x, greater than that of the isolated litter, whereas when suckling the rat with lesions it gained at a net rate of y greater than that of the isolated litter, then the lactational performance in percentage of normal of the rat with lesions would be (y/x)×100.

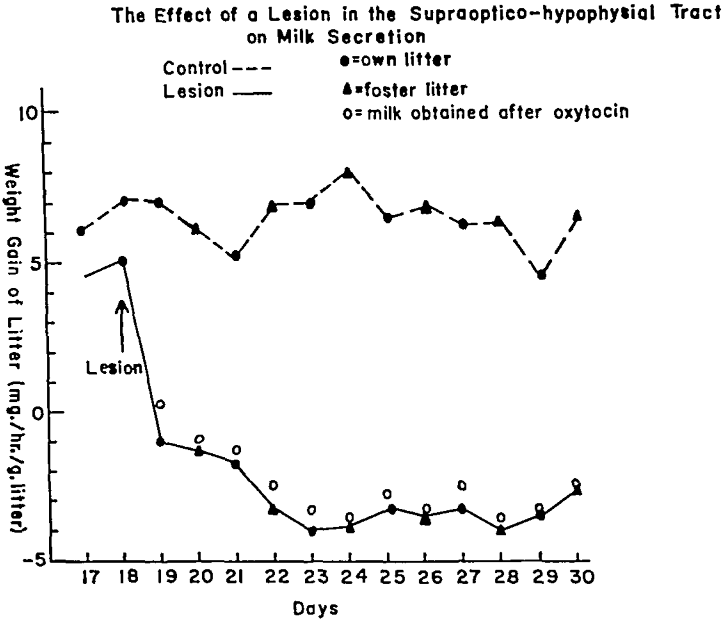

FIG. 1

"Small" lesions (Table 3A) produced a similar picture, but the reduction in milk production appeared to be not quite as pronounced, the mean secretion being 21% of normal (Table 3A, Fig. 3). The breasts of these animals exhibited less involution than that seen in rats with "large" lesions. The results obtained in one of these cases are illustrated in detail in Figure 2. It is noteworthy here that, after an initial decline in litter weight gain, there was a rebound toward normal from the third to the seventh day, by which time the milk yield had once again fallen to very subnormal levels, where it remained for the remainder of the experiment. This rebound increase in milk yield for a short time during the first post-operative week, noted also in rats with large lesions, was seen altogether in 7 out of the 13 cases with a deficiency of oxytocin release.

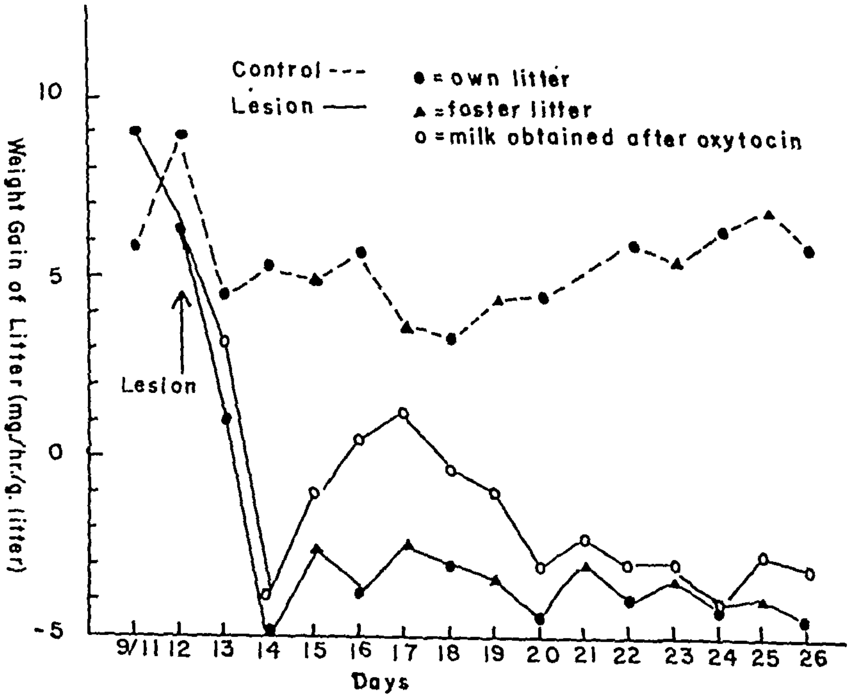

FIG. 2. The effect of a "small" lesion in the supraoptico-hypophysial tract on milk secretion

It is surprising that the weight loss in the absence of oxytocin in litters suckling rats with lesions often exceeded that found when the litters were isolated and no intake occurred (Tables 1 and 3A). This discrepancy may be related to a higher environmental temperature when with the mother, which produced a greater insensible loss of water. Exercise during suckling efforts may have also contributed to a greater weight loss. Lastly, diuresis induced by ingestion of milk following the preceding injection of oxytocin would increase the rate of weight loss of the litter. This factor is relatively small since the amount of milk obtained following the last oxytocin injection was usually very small.

A relationship between endogenous antidiuretic hormone (ADH) release and ACTH secretion has been demonstrated in rats with hypothalamic lesions (19). This later relationship was obtained by plotting water intake as an index of the severity of diabetes insipidus and thereby deficiency of ADH versus ACTH release. An attempt to relate water intake

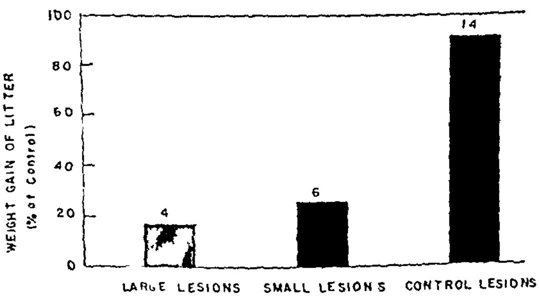

FIG. 3. The Effect of Hypothalamic Lesions on the weight gain of litters. The height of each bar represents the mean effect of each type of lesion. The number above each bar represents the number of mother rats with that type of lesion.

to milk secretion is shown in Figure 4, which illustrates that in the present experiments there was no correlation between the presence of a deficiency of ADH release, as indicated by polydipsia, and milk secretion. Rats with a near maximal impairment in milk secretion were seen to have no diabetes insipidus, whereas other animals with definite diabetes insipidus had essentially normal milk secretion. Furthermore, it is equally apparent (Tables 2 and 3) that milk ejection (i.e., weight gain in the absence of oxytocin) was also unrelated to polydipsia.

There was no correlation between the deficit in lactation and ovarian or adrenal weights, though occasionally these weights were definitely subnormal (Tables 2 and 3). Impaired lactation was likewise seen in the presence of normal sized uteri. Furthermore, since the weight loss which was often seen in mothers after lesions interrupting lactation was no greater than that seen after control lesions, it appears that changes in nutritional status cannot account for the results.

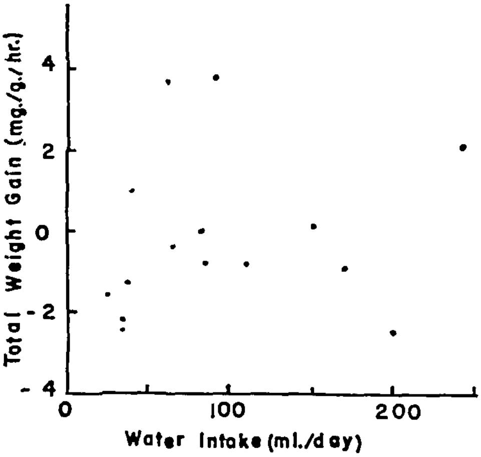

FIG. 4. The failure of litter weight gain to correlate with water intake, indicating that milk secretion in the rat with hypothalamic lesions is unrelated to diabetes insipidus.

Serial sections of diencephalon and pituitary were made in all rats with marked impairment in lactation (Table 3B), and in all but 2 of the 14 control rats. In all cases where a defect of milk ejection and milk secretion was present the lesions were seen to produce at least a partial interruption of the supraoptico-hypophysial tract as judged by their location, by an atrophy of the infundibular process, and by a decrease in the number of supraoptic neurons (Table 3B). In 2 cases where it was studied, there was a disappearance of neurosecretory material from the infundibular process. In one of the two cases (Rat 5) where a less complete impairment of milk ejection and lactation was seen, it was apparent from the sections that the interruption of this tract was less complete than in the other cases. This was not surprising in this case since the lesions had been designed to reach the base of the hypothalamus just lateral to the supraoptico-hypophysial tract. However, in rat 9B the other rat with a less pronounced deficit, the supraoptico-hypophysial tract had sustained considerable injury. A less complete injury to this tract did not block milk ejection or lactation in rats P-1, P-4, 6, 4 and 12 (Table 2). It is of interest that preliminary observations suggest that loss of neurosecretory substance from the neural lobe appears to be a very sensitive index of injury to the supraoptico-hypophysial tract in lactating rats since the material was depleted from 6 neural lobes where the injury to this tract was minimal and lactation was not blocked, whereas in 6 rats where the tract was spared, there was abundant neurosecretory material in the neural lobe. Apparently, in the presence of injury to the tract reducing the reserve of secretory tissue, function may be maintained but at the expense of storage of neurosecretory material.

TABLE 3B. LOCATION OF LESIONS IN RATS WITH INJURY TO THE SUPRAOPTICO-HYPOPHYSIAL TRACT

	Structure injured (graded as to severity from + to ++++)						
Rat No.	Median eminence, M.E.	Portal in M.E.	Veins in stalk	Supra-optic nucleus[1]	Paraven-tricular nucleus[2]	Atrophy of neural lobe	Supra-optico-hypo-physial tract
1. "Large" Lesions							
4	++++	++++	0	+++	++	++	+++
1D	+++	+++	0	++	++	++	++
2D	+	+	0	++	+++	++	++
4D	++	+++	0	++	+++	+	++
2. "Small" Lesions							
2B	+	+	0	+++	+++	+++	+++
5B	++	+	0	+++	+	−−−	+++
6B	+++	+++	0	++	++	++	+++
7B	+	+	0	+++	++	−−−	+++
8B	+	+	0	++	++	++	++
A5	+++	+++	0	++++	++	+++	+++
A4	+	+	0	++	+	+	++
3. Partial Lesions							
9B	++	++	0	++	+	++[4]	++
5	0	0	0	+−++[3]	+−++	+[4]	+−++

[1] Injury restricted to retrograde degeneration of neurons.
[2] Direct injury from electrode tracts, and retrograde degeneration.
[3] Almost complete unilateral destruction.
[4] Complete loss of neurosecretory material.

In the rats with deficient oxytocin release and milk secretion, the interruption of the supraoptico-hypophysial tract occurred either in the median eminence or just in front of this structure. In 7 rats it was damaged more or less severely, while in 6 rats the eminence appeared to be completely, or nearly completely, intact. It, therefore, appears that injury to the median eminence itself or structures in its immediate vicinity is not a necessary condition for a defect in lactation. Specifically, it is unlikely that injury to fiber tracts entering the eminence from the posterior hypothalamus contributed to the effect or that injury to portal vessels, pars tuberalis, or arcuate nuclei played a role. A section through the intact median eminence of rat No. 7 B is shown in Figure 5. Transverse sections through the hypothalamus of 4 of the rats in which the median eminence was spared are illustrated in Figure 6.

Study of the anterior lobes of the rats with lesions revealed that the vascular supply to the gland was abundant and no infarcts were seen.[4] In one case, rat 4 (Table 3A), the anterior lobe was definitely smaller than normal whereas in the other cases it appeared to be about as large as that of the control rats; however, no volume determinations have as yet been made. All anterior lobes contained eosinophiles, basophiles, and chromophobes, but no attempt has been made to determine if abnormalities in the numbers of these forms were present.

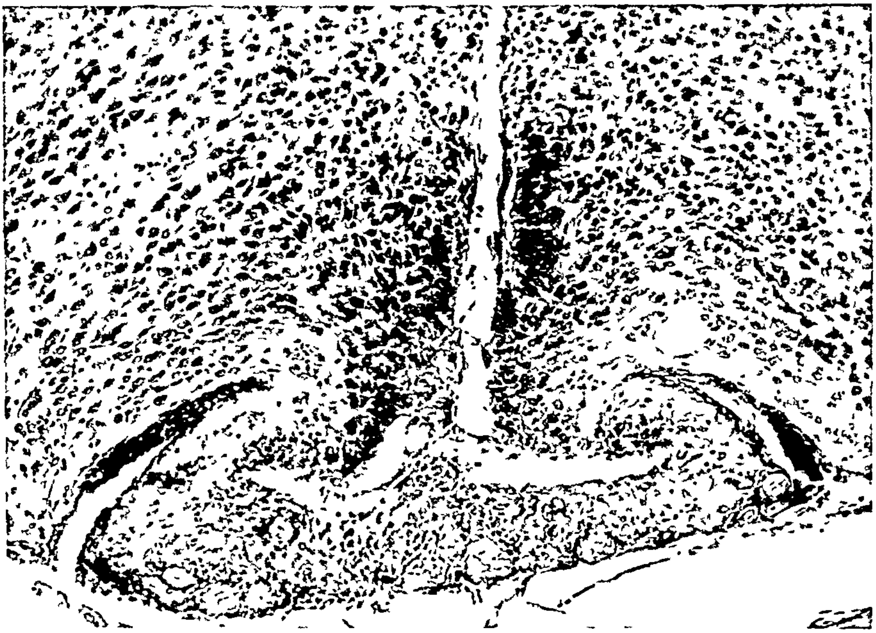

Fig. 5. A transverse section through the median eminence of one of the rats with a "small" lesion in the hypothalamus demonstrating intact hypophysial portal veins filled with red cells.

II. *The Effect of Posterior Lobe Extracts on the Course of Mammary Involution in the Rat*

A. *Normal Rats.* After removal of the litter, the rat's mammary glands involute. There is a failure of secretion, followed by a loss of alveolar structure, and a conversion of parenchyma into fat (6, 11). This process is nearly complete nine days after removal of the litter in the saline injected rats (Table 4). In contrast, injections of Pitocin, a commercial extract of oxytocin, were able markedly to retard the involution. There was much more parenchymal tissue in the mammae from oxytocin-injected rats. Furthermore, there was a considerable preservation of alveoli, and secretion was present. In some cases alveoli and ducts were abnormally distended with secretory material; however, these glands were usually not in a lactational condition. The effect was then a retardation but not a prevention of involution. The minimal effective dose of Pitocin to produce a retardation of involution appeared to be about 0.25 u., I.P., three times daily.

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[4] In earlier experiments with lesions located more posteriorly in the median eminence, infarcts similar to those seen after stalk section were noted (21). These partiuclar rats with lesions also had considerable apparently well vascularized pituitary tissue and demonstrated lactational findings like those of rats with the "large" lesions reported here. Because of the question as to the state of anterior lobe vascularization, these rats were not included in the present report.

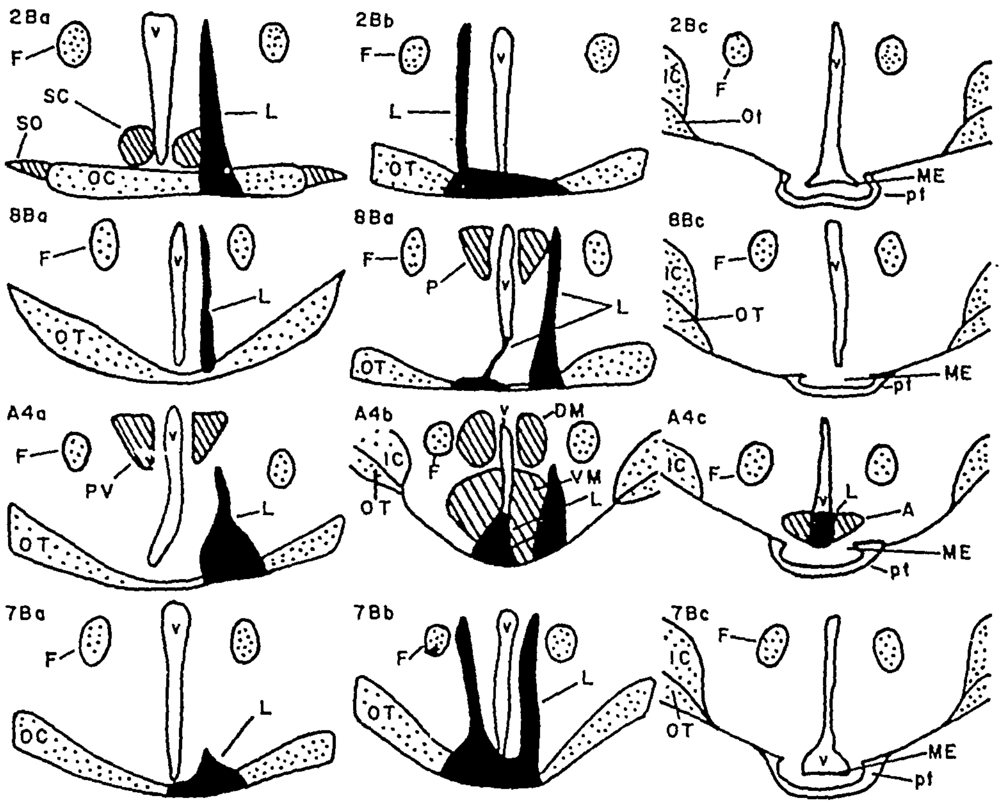

FIG. 6. Drawings of transverse sections through the hypothalamus of four of the rats with "small" lesions. Sections marked "a" pass through the rostral tip of the lesions; those marked "b" pass through the greatest extent of the lesions; and those marked "c" are just caudal to or at the caudal tip of the lesions.

Key for abbreviations:

IC—Internal capsule
CC—Optic chiasm
OT—Optic tract
F—Fornix
V—Third ventricle
A—Arcuate nucleus
DM—Dorsomedial nucleus

PV—Paraventricular nucleus
SC—Suprachiasmatic nucleus
SO—Supraoptic nucleus
ME—Median eminence
pt—Pars tuberalis of pituitary
L—Lesions

Pitressin, a commercial extract of vasopressin, produced a definite though less marked effect (Table 4). Its potency appears to be roughly half that of oxytocin, though more work is required to establish this. Examples of the effects of Pitocin and Pitressin are shown in Figures 7 and 8. Syntocinon, a preparation of synthetic oxytocin, appeared to be as active as Pitocin in inhibiting mammary involution (Table 4).

B. *Rats with Hypothalamic Lesions.* Since oxytocin produces milk ejection by inducing contraction of myo-epithelial elements in the breasts, it was possible that this contraction produced afferent impulses which would reflexly cause stimulation of lactogenic hormone release by an unknown mechanism and thereby a retardation of involution of the breast. A reflex type of retardation has already been demonstrated to be produced by suckling or application of turpentine to nipples (6, 21). It seemed of interest to determine if oxytocin could still retard mammary involution in the presence of a hypothalamic lesion. Although the mammary involution in the saline injected rats with hypothalamic lesions was not as complete as seen in the normals (Table 4), mammary involution was significantly retarded by either Pitocin, or synthetic oxytocin (Table 4, Fig. 9). Serial sections of hypothalamus and pituitary of the seven rats injected with synthetic oxytocin revealed that all of these lesions were similar to those

TABLE 4. RETARDATION OF INVOLUTION OF THE BREAST OF RATS PRODUCED BY POSTERIOR LOBE EXTRACTS

Treatment	Response[1]	
	Individual	Mean
A. *Normal Rats*		
1. Saline	0, 0, 0, 0, 1, 0, 0, 0, 0, 1	0
2. Pitressin,		
1.0 U.,[2] t.i.d.[3]	1, 2, 2, 2, 2, 1	2−
0.5 U.,[2] t.i.d.[3]	0, 1, 2, 2	1
3. Pitocin,		
1.0 U., t.i.d.	2, 2, 3, 1, 2, 4, 2	2+
0.5 U., t.i.d.	1, 3, 2, 2	2
0.25 U., t.i.d.	1, 1, 2, 0	1
4. Synthetic Oxytocin		
1.0 U., t.i.d.	2, 2, 2, 1, 3	2
B. *Rats with Hypothalamic Lesions*		
1. Saline	1, 1, 2, 1, 2, 1 2, 1, 2, 0, 1, 2	1
2. Pitocin		
1.0 U., t.i.d.	3, 4, 2, 3, 1, 3	3−
3. Synthetic Oxytocin		
1.0 U, t.i.d.	4, 3, 4, 4, 2, 3, 3	3

[1] Responses graded from 0–4; 1–3 = progressively decreasing degrees of involution; 4 = lactational condition.

[2] U. = International unit.

[3] t.i.d. = Thrice daily.

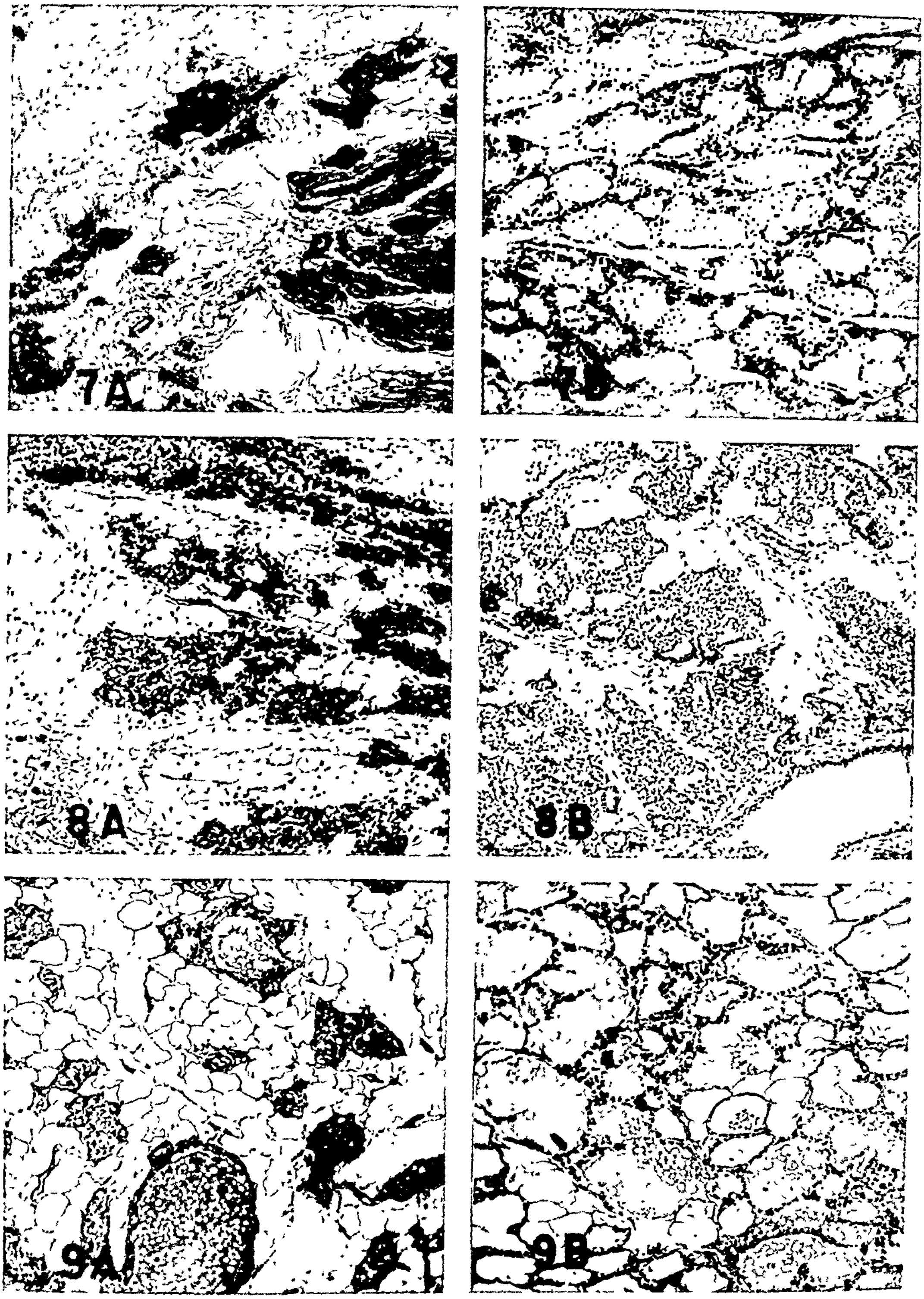

FIG. 7–9. Microphotographs at the same magnification of sections of breasts from rats whose litters had been removed 9 days previously.

FIG. 7A. Section from a control rat injected thrice daily with saline. Note that marked involution of the breasts has taken place leaving only small islands of parenchymal tissue, consisting of cords with only occasional tiny alveoli (Grade=0).

already described and that they produced at least a partial interruption of the supraoptico-hypophysial tract.

DISCUSSION

The present experiments are the first to our knowledge to demonstrate a failure of milk secretion after hypothalamic lesions. This marked deficit in milk secretion was associated with a failure of milk ejection, indicative of a deficiency of endogenous oxytocin release.

It might be argued that the apparent deficit in milk production found in these rats with lesions was due to an inability of infants to obtain all the milk which was available in the breasts when effective nursing was limited to 1–3 periods a day shortly after an injection of oxytocin. This is not the case since, even after a single shot of oxytocin, fasted litters have been observed to gain at least 10% of their body weight (see section I A). This would mean that, if oxytocin were injected only once a day, the weight gain following oxytocin could equal at least 4.2 mg./g./hr. Such a weight gain was achieved in only one of the litters of the present study (Table 3A, rat 9B), and this mother rat was injected thrice daily with oxytocin. The other litters in the study, many of them also receiving the benefit of three injections of oxytocin, gained from 3.2 down to a minimum of 0.6 mg./g./hr. after oxytocin. The results, therefore, indicate that there is a deficit in milk yield in rats with a deficiency of oxytocin release.

Since limitation of suckling to 8 hours a day has been found to produce a marked reduction in litter weight gain in normal rats (McCann, unpublished), it is conceivable that the observed defect in lactation in rats with lesions might be due to decreased suckling time of litters. This possibility can not be completely ruled out; however, it seems an unlikely one since

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FIG. 7B. Section from a rat injected thrice daily with Pitocin. Note the preservation of alveoli containing secretion (Grade = 4)

FIG. 8A. Section from another control rat injected thrice daily with saline showing essentially the same degree of involution as seen in Figure 7A (Grade = 0).

FIG. 8B. Section from a rat injected with Pitressin thrice daily. Note that, although involution has progressed considerably, alveoli persist which are considerably larger than in the control rats. A large duct containing some secretion can be seen in the lower right hand corner which also distinguishes this section from the controls (Grade = 1).

FIG. 9A. Section from a control rat with a "small" lesion in the supraoptico-hypophysial tract injected thrice daily with saline. Note that, although there is little parenchyma present, there are several fairly large alveoli and two large ducts containing secretion, so that involution has not progressed quite as far as in saline-injected normal rats (Grade = 1)

FIG. 9B. Section from a rat with a "small" lesion in the supraoptico-hypophysial tract injected thrice daily with Pitocin. Many large secretion filled alveoli are present though some alveoli show beginning involution (Grade = 4).

there is no evidence for failure of suckling. Mothers with lesions were observed to suckle their infants as much as normal rats or those with control lesions. In fact, it was notable that in spite of failure to obtain milk, the litters were almost always attached to the teats and that the mothers with lesions retrieved their litters in a normal manner when they were taken from the nest, apparently manifesting normal maternal behavior.

If one grants then that the observed failure of lactation was in fact due to a failure of milk secretion, it would appear probable that this defect was due to the loss of the constellation of pituitary hormones which are required for maintainance of lactation in the rat. That some pituitary hormones were still being secreted by the rats with lesions is suggested by the fact that the deficit in lactational performance in rats with hypothalamic lesions was less than that which followed hypophysectomy in the experiments of Cowie (22).

Since adrenalectomy has an adverse effect on lactational performance in the rat which can be corrected by cortical hormones (23), it might be argued that the defect in lactation following lesions was due to decreased ACTH output by the pituitary. It is known that lesions similar to those reported here can prevent the increased ACTH release which follows acute stress (1–3); however, since complete adrenalectomy produces a much less marked defect in lactation than that reported here, it appears that diminished ACTH secretion alone could hardly account for the present results. Since ovariectomy has little effect on lactational performance in the rat (23), it appears very unlikely that deficits in ovarian secretion secondary to diminished gonadotrophin secretion could explain the results. As growth hormone is capable of increasing milk yields of hypophysectomized rats injected with prolactin (22), it is possible that alterations in growth hormone release may have played a part in the results. Because injections of prolactin are most effective in restoring lactation in the hypophysectomized rat (12), it would appear most likely that the present results were due to a deficiency in the output of pituitary lactogenic hormone (prolactin) and possibly ACTH and growth hormone as well.

The rebound increase in milk yield noted in some of the rats during the first postoperative week is reminiscent of the "normal interphase" of diabetes insipidus. This latter is supposedly due to the release of ADH from the distal ends of the cut fibers of the supraoptico-hypophysial tract (24). Could a similar release of the neurohumor regulating pituitary lactogenic hormone secretion account for the findings in these lactating rats with lesions?

Benson and Cowie (18) have reported that posterior lobectomy abolishes milk ejection in the rat, but that injections of oxytocin permit withdrawal of milk sufficient to sustain litter growth at a rate only slightly below normal. In these experiments, in contrast to those reported here, there was a dissociation between the lack of milk ejection and milk secretion. This may be accounted for by the possibility that in their experiments there was a secretion of neurohumor from the median eminence into the hypophysial portal vessels. This neurohumor reaching the anterior lobe in high concentration via the portal vessels might be sufficient to stimulate lactogenic hormone release, whereas when it reached the mammary glands after being diluted in the systemic circulation, it would be incapable of inducing milk ejection. Consistent with this idea that some neurohumor (oxytocin) secretion might have still occurred after removal of the neural lobe in their animals is their finding that after remating and parturition no deficiency of milk ejection was present in the succeeding lactation.

Cross and Harris (5) reported a block of milk ejection in rabbits after lesions in the supraoptico-hypophysial tract but found that milk secretion was not greatly affected, again in contradistinction to the present results. Rats apparently differ from rabbits in that in rats the suckling stimuli must continue over most of the 24 hour period to sustain normal milk secretion, whereas Cross and Harris (5) and later Donovan and Van der Werff ten Bosch (25) found normal growth of litters of rabbits allowed to suckle only once daily. It is also possible that the difference between their results and those reported here may have been due to a less complete interruption of the supraoptico-hypophysial tract in their experiments. Recently, Donovan and Van der Werff ten Bosch have similarly reported no interference with milk secretion in rabbits with hypothalamic lesions (25), whereas pituitary stalk section often left milk yield normal but poor in quality. Here, oxytocin was always given to the rabbits with lesions prior to the nursing period of the litter, making it impossible to judge on physiological grounds whether or not a deficiency in the secretion of oxytocin was actually present in their animals.

The results with oxytocin and Pitressin confirm the findings originally reported by Benson and Folley (11) that these materials delay mammary involution. Oxytocin appears to have the major action in this regard though the action of Pitressin is also considerable. It appears likely that the vasopressin molecule may have some intrinsic activity in this regard as analogously vasopressin possesses one fifth the activity of oxytocin in evoking milk ejection (26). It has previously been demonstrated by Bradley and Clarke (27) that injection of oxytocin directly into the ductile system of the breast of rabbits has no stimulatory effect in contrast to the known direct stimulatory action of lactogenic hormone (28). Furthermore, Benson and Folley have shown that oxytocin has no action in delaying involution of the breast in the hypophysectomized rat (11). Consequently, it appears that this action of oxytocin is not exerted locally on the breast and that it requires the presence of the pituitary. Since lactogenic hormone injections produce a similar retardation of involution (9), it is reasonable to suppose that the action of oxytocin is exerted by stimulating a release of prolactin. As the effect of oxytocin persists in the rat with hypothalamic lesions which should block any neural control of lactogenic hormone secretion, it appears likely that oxytocin stimulates the release of lactogenic hormone from the pituitary by a direct action on this gland.

The explanation for the slightly diminished involution of the breast found in the saline-injected rat with lesions is not known. It is conceivable that this represents residual lactogenic hormone secretion in the rat with a lesion. Everett (29) has described persistent luteotrophin secretion in hypophysectomized rats bearing pituitary grafts.

In the rat it appears that pituitary lactogenic hormone and luteotrophin may be identical (30). If this be so, then oxytocin should also stimulate the release of luteotrophin. Indeed, it has already been reported that oxytocin can induce pseudo-pregnancy in the rat (31, 32), and Benson and Folley reported mucification of the vagina in their oxytocin-injected rats (11). Part of the effect of oxytocin in delaying mammary involution may be due to progesterone secretion.

Grosvenor and Turner (33) have reported that oxytocin injection results in a relatively small decrease in pituitary prolactin content when compared to the decrease that follows suckling. They have interpreted this result as being contrary to the thesis that oxytocin stimulates prolactin release. Our results also indicate that the doses of oxytocin injected did not replace the effect of normal suckling stimuli by any means completely. The breasts of oxytocin injected animals were usually not in a fully lactational condition and oxytocin injections did not return lactation to normal in rats with lesions.

As in the case of the effect of vasopressin on ACTH release (1–3), the dose of oxytocin required to produce apparent lactogenic hormone release from the pituitary appears to be much larger than the dose required to produce effects in the periphery, in this case milk ejection. Oxytocin may still play a physiological role in stimulating the pituitary if it reaches the gland in sufficiently high concentration after secretion into the hypophysial portal vessels. Alternatively, it is possible that the effects of oxytocin and vasopressin on the breast occur only at pharmacological doses and that the true neurohumor may be a closely related polypeptide.

Since a deficiency of oxytocin leads to a deficiency of milk secretion presumably mediated by a decrease in lactogenic hormone release, whereas injections of oxytocin appear capable of stimulating lactogenic hormone release by a direct action on the pituitary, it is suggested that oxytocin may be the neurohumor which stimulates the release of lactogenic hormone, or the complex of hormones which maintains lactation. Much more work is necessary to establish this hypothesis.

A surprising incidental finding in the present study was the lack of correlation of oxytocin release as evidenced by milk ejection and ADH release as determined by polydipsia. This could be considered evidence for a view that ADH and oxytocin are synthesized and released by different neurons of the supraoptico-hypophysial tract. Olivecrona (17) has already suggested that the paraventricular nuclei preferentially secrete oxytocin; however, so far we have no evidence in favor of this particular concept. Rather, it would seem more likely that the cells of both the supraoptic nuclei and paraventricular nuclei secrete either oxytocin or ADH (36).

Acknowledgment

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INHIBITION OF THE DEVELOPMENT OF THE BURSA OF FABRICIUS IN THE EMBRYOS OF THE COMMON FOWL BY 19-NORTESTOSTERONE[1]

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ABSTRACT

0.63 mg. of 19-nortestosterone in 0.1 ml. of corn oil, when injected into the fertilized eggs of the white leghorn hybrid strain of chickens, "Wisco-white," on the 5th day of incubation, completely inhibits the development of the bursa of Fabricius in both sexes. In the normal chick the bursal primordium appears as a proliferated epithelial mass dorso-caudal to the embryonal cloaca on the 5th day of incubation. By 168 hours of incubation it forms a tubular structure; its terminal end enlarges into a bursal vesicle on the 10th day and becomes lymphoidal by the 15th day. The bursa is fully formed and lymphoidal in 17-day old chick embryos. In 19-nortestosterone-treated embryos the bursal primordium becomes highly vacuolated. Later it remains as a convoluted, short duct without forming the bursal vesicle. Thus the hatched chicks are bursaless. The secondary effects of this hormone on the rectal complex are the stunted growth of the ventral anal lip, increase of musculature in the dorsal lip and an attenuated proctodial canal with very few anal mucoid glands.

IT IS known that the bursa of Fabricius and other lymphatic tissues are considerably reduced by the administration of androgens in young birds (1, 2, 3, 4). Meyer, Aspinall and Buchanan (5) found that when the androgen, 19-nortestosterone, was injected into the egg on the 5th day of incubation and the chicks examined grossly at 19 days or post-hatching, there was a complete absence of the bursa of Fabricius as well as a reduction in size of the thymus and spleen. This discovery provided the stimulus for making a histological study of the organogenesis of the bursa in 19-nortestosterone treated and normal chick embryos. It is the objective of this report to describe the results of this investigation.

MATERIALS AND METHODS

In the chick the bursal primordium appears as a proliferation from the dorso-caudal epithelium of the embryonal cloaca on the 5th day of incubation (6). On this day 0.63 mg. of 19-nortestosterone in 0.1 ml. of corn oil was injected at the pointed end of the fertilized egg and the opening in the shell was sealed by paraffin or collodion. Eggs injected with 0.1 ml. of corn oil were incubated with the hormone-treated eggs. The eggs were from a white leghorn hybrid strain of chickens, the "Wisco-white."

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One treated and one control embryo were removed at 8-hour intervals from 120 to 264 hours and thereafter two treated and two control embryos were selected at each 24-hour interval through day twenty-one. Seventy-eight embryos representing 29 stages were made available by this method of selection. One control and one hormone-treated embryo for each day were studied in detail, a total of 58 embryos.

The caudal portions of these embryos were preserved in Bouin's fixative and processed in the usual manner. Sagittal sections of 10 μ thickness were cut and stained in Harris' haematoxylin-eosin.

It was possible to determine the sex of the embryos by visualization of gross characteristics of the gonads only after 11 days of incubation. Thus in 22 treated embryos 10 were males and 12 were females. In 21 control embryos 12 were males and 9 were females.

OBSERVATIONS

Origin and vacuolization of the bursa of Fabricius

a) *128 hours of incubation:* At this stage the bursal primordium in the normal embryos appears as a proliferation of the endodermal epithelium in the dorso-caudal region of the embryonal cloaca (urodeum). Small vacuoles are present in its dorsal aspect (Fig. 1). The origin of the proctodeum is indicated by a caudal depression which is lined by columnar epithelium.

In 19-nortestosterone treated embryos the bursal primordium shows many large vacuoles distributed both dorsally and ventrally (Fig. 2).

b) *168 hours of incubation:* The vacuoles of the dorsal region of the bursal primordium fuse to form a bursal vesicle with a duct communicating with the proctodial canal in the normal chick (Fig. 3). The bursal vesicle and the duct are lined by two to three layers of columnar epithelium, whereas the proctodial canal is lined by cuboidal or flat cells. The vacuoles in the ventral region of the bursal primordium gradually disappear. The proctodial canal is deep and is bounded by dorsal and ventral anal lips which are well developed and are of the same size. The embryonal cloaca extends into the ventral anal lip (Fig. 3).

In 19-nortestosterone treated chicks, the bursal primordium persists as a mass of cells with large ventral vacuoles into which the proctodial canal opens. There is no formation of the bursal vesicle and duct. The proctodial canal is shallow and wide where the proctodeum opens into the ventral aspect of the vacuolated bursal mass. The ventral anal lip is considerably smaller than the dorsal anal lip (Fig. 4).

Growth of the bursal vesicle and plication

In the normal chick, the bursal vesicle, which was narrow in the previous stage, enlarges into a large vesicle after 216 hours of incubation. At 240 hours of incubation the bursal vesicle is large and oval extending dorso-anterior to the embryonal cloaca. Folds arise from the vesicle due to the ingrowth of connective tissue (Fig. 5), and in these folds the cells are loosely arranged and are undergoing mitosis. Some of the epithelial cells are transforming into lymphocytes wherein there is condensation of chromatin. The bursal duct is narrow and long, communicating with the

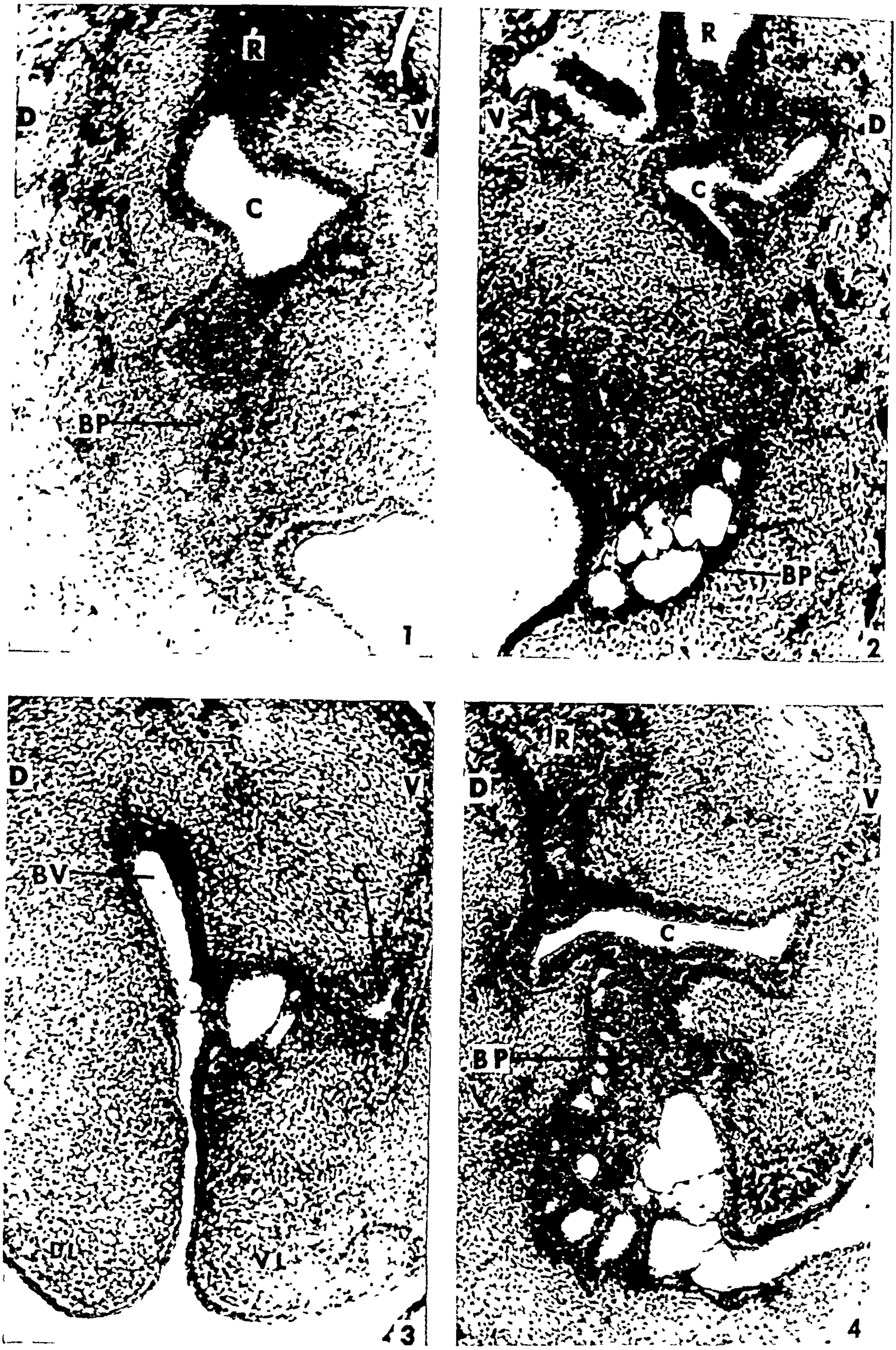

PLATE 1

Sagittal sections of the rectal region of the chick embryos. Fixative: Bouin's solution. Stain: Harris' haematoxylin-eosin.

proctodial canal which is lined by cuboidal cells. The proctodial canal is deep and is bounded by dorsal and ventral anal lips of equal size (Fig. 5).

The bursa in 19-nortestosterone-treated chick embryos is a narrow tube which opens into a narrow, short proctodial canal bounded by dorsal and ventral anal lips of unequal size. The ventral anal lip is short and stumpy (Fig. 7). Surrounding the terminal end of the bursal duct is a diffuse mass of epithelial cells which appears as a dark area (Fig. 7) representing the region of the bursal vesicle. The bursal duct is lined by columnar epithelium which is pseudostratified, especially at the terminal end.

Development of lymphoid follicles in the plicated bursal vesicle

On the 12th day of incubation the entire bursal vesicle in the normal chick is very plicated and solid epithelial buds arise from the folds. In 13 and 14 day old embryos the buds are increased in size and number. During the 15th day these buds are pinched off and many of them are becoming lymphoidal in nature. The bursa in 17 day old normal embryos is large and plicated, with many lymphoidal follicles (Fig. 6). Many eosinophils are found in the vicinity of these follicles. The bursal duct is narrow and communicates with a long and narrow proctodial canal, which is bounded by well developed dorsal and ventral anal lips (Fig. 6). Its ventral wall is thrown into folds while many anal glands are arising as epithelial buds from its dorsal wall (Fig. 6). Both the lips are approximately of the same size and there is slight bend in the dorsal anal lip.

In the 19-nortestosterone-treated embryos the bursal duct is highly convoluted, narrow and surrounded by muscular dorsal anal lip and a narrow, stumpy ventral lip. Unlike normal chicks there are very few small anal glands. These arise from the dorsal wall of the bursal duct (Fig. 8). The rectum, at this stage, has enlarged considerably and is filled with secretions (Fig. 8).

Generalized development of the bursa and proctodeum occurs between

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FIG. 1. 128 hours of incubation in the normal embryo showing the formation of the bursal primordium. Note the vacuoles in its dorsal aspect. ×114.

FIG. 2. 128 hours of incubation, 8 hours after administration of 19-nortestosterone. Note the presence of large vacuoles in the bursal primordium. ×114.

FIG. 3. 168 hours of incubation—Normal. The bursal tube is formed communicating with the proctodial canal. The dorsal and ventral anal lips are of the same size. ×114.

FIG. 4. 168 hours of incubation—Treated. The vacuolated bursal primordium persists. The proctodial canal is wide and shallow, communicating with the ventral aspect of the bursal primordium. ×114.

Abbreviations

B—Bursa of Fabricius.
BD—Bursal duct.
BP—Bursal primordium.
BV—Bursal vesicle.
C—Embryonal cloaca (Urodeum)
D—Dorsal region.
DL—Dorsal anal lip.
R—Rectum.
V—Ventral region.
VL—Ventral anal lip.

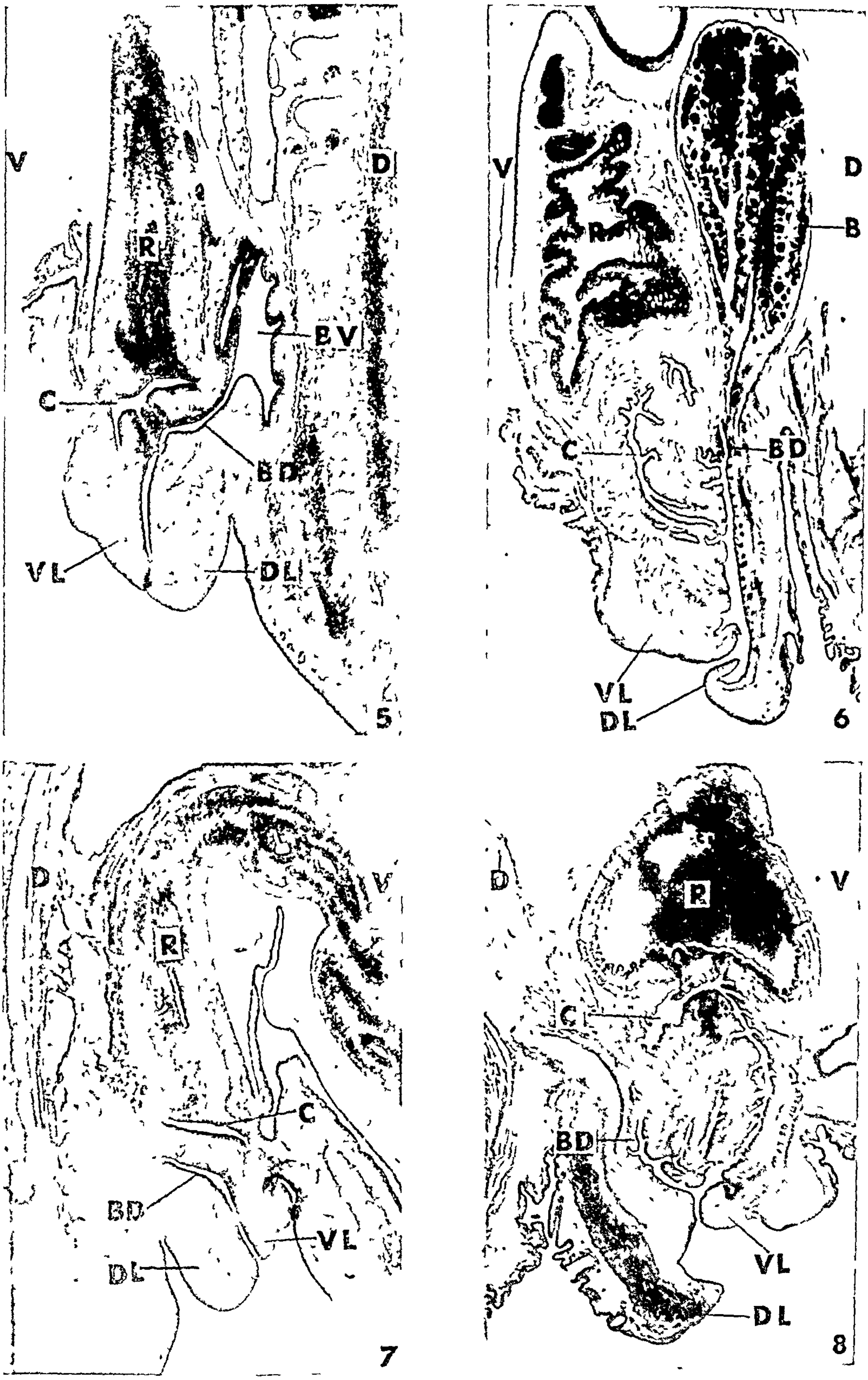

PLATE 2

Sagittal sections of the rectal region of the chick embryos. Fixative: Bouin's solution. Stain: Harris' haematoxylin-eosin.

the 18th and 21st day in normal chicks. The mucoid anal glands also increase in number and size. The bursa, however, is greatly attenuated to a short convoluted duct in 19-nortestosterone-treated chicks. The proctodial canal is short and narrow, bounded by a muscular dorsal anal lip and a short, stumpy ventral anal lip. Very few mucoid anal glands develop. The rectum is large and thin-walled.

DISCUSSION

Effect of 19-nortestosterone in suppressing the development of the bursa of Fabricius

Whether male gonadal hormones are lympholytic or inhibit the formation of new lymphocytes is still debated. Since there is accelerated destruction of these cells in intact animals as compared to gonadectomized animals, Dougherty (7) thought that androgens were lympholytic. Brauchhardt (8) noted that testosterone propionate inhibited mitosis of the epithelial cells of the intestine of guinea pigs and causes considerable reduction in lymph node weight. Agrel and Persson (9) indicated that testosterone propionate inhibited the formation of desoxypentose nucleic acid and correspondingly blocked cellular cleavage.

Histological changes of the bursa in treated embryos show that in the initial stages there is vacuolization of the bursal primordium (Figs. 2 and 4), but macrophages are not present, nor is there any degeneration of epithelial cells in that area as seen in the involuting lymphoid tissues. In the later stages the bursa remains as a convoluted, short duct and the bursal vesicle does not develop (Figs. 7 and 8). It is interesting to note that irrespective of the sex the bursal development is inhibited by 19-nortestosterone. This may be attributed to the suppression of the mitotic activity of the cells in the terminal region of the bursal duct, thus confirming the views held by Brauchhardt (8) for the thymus and other lymphoid tissues.

In another series of experiments, the results of which will be described in another report, it was found that in the newly hatched chicks treated with 19-nortestosterone on 5th day of incubation, the thymus and spleen,

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FIG. 5. 240 hours of incubation—Normal. The bursal vesicle is fully formed within which folds are appearing. Dorsal and ventral anal lips are of the same size. ×21.

FIG. 6. 17 days of incubation—Normal. The bursa is fully formed with many lymphoid follicles. The proctodial canal is long, bounded by well developed dorsal and ventral anal lips. Note the presence of many anal glands along the dorsal wall of the proctodial canal. ×13.

FIG. 7. 240 hours of incubation—Treated. The bursal vesicle is absent and that region is represented by a mass of epithelial cells stained dark at the terminal end of the bursal duct. The ventral anal lip is short and stumpy. ×21.

FIG. 8. 17 days of incubation—Treated. The bursa is absent, and the bursal duct is greatly reduced. The proctodial canal is short and narrow. A few small anal glands are present in the basal region of the bursal duct. Note the well developed musculature in the dorsal lip and the short, stumpy ventral lip. ×13.

although present, were reduced 30 to 50% when compared to those of non-treated chicks.

Although the present experiment was designed to suppress the development of the bursa in the chick, there were some secondary effects of this hormone on the rectal complex. Both dorsal and ventral anal lips were long and well developed in the normal chicks and the proctodial canal was long and lined by many large mucoid glands (Figs. 5 and 6). In 19-nortestosterone treated chicks there was, however, unequal growth of these lips; the ventral anal lip was short and stumpy but the dorsal lip was highly muscular. The latter may have been due to the myotrophic effect of this hormone and the former due to the delay in the growth of the posterior extension of the cloaca (Figs. 4, 7 and 8). This delay in the growth of the posterior extension of the embryonal cloaca may be due to the retarded growth and to the presence of the vacuolated bursal primordium in the posterior region of the cloaca (Fig. 4). Consequently, the proctodial canal was narrow and short with very few anal mucoid glands. It is suggested that this caused the feces to accumulate in the cloaca, thus plugging the vent. The plugging may be due in part to the short ventral anal lip and muscular dorsal lip and to the insufficient production of mucous secretion which acts as a lubricant in the normal chick. These effects were seen in the pheasant as well as in the chickens (10).

Significance of this experiment

The function of the bursa of Fabricius is still debated as bursectomy has no influence either on the somatic growth or on the gonads except for a slight increase in the size of the ovary (11, 12, 13, 14, and 3). Riddle and Krizenecky (11) could not observe any appreciable effect after bursectomy and thymectomy in body growth and gonadal activity in pigeons. These lymphoid tissues are so widely distributed that bursectomy and thymectomy are without appreciable effect. Gyllensten (15) and Gyllensten and Ringertz (16) have shown that partial thymectomy in guinea pigs causes hyperplasia of other lymphoid tissues. Recently, Metcalf and Buffett (17) have shown in normal, thymectomized and adrenalectomized mice a lymphocytosis response after the administration of thymic extract which, they believe, is caused by some "lymphocytosis stimulating factor" present in the thymus and not in the lymph nodes. Comsa (18) and Comsa and Leroux (19), however, believe that in guinea pigs the lymphocytosis caused by thymus extract is mediated through the adrenal in which there is hyperplasia of the medulla and a considerable reduction in the cortical region. Glick (3) indicates that bursectomy reduces the production of antibodies. Therefore, a complete suppression of the bursa with sufficient atrophy of other lymphoid tissues, especially thymus, by 19-nortestosterone may open further avenues for elucidating the above mentioned problems, especially the function of the bursa and other lymphoid tissues in general.

SUMMARY

0.63 mg. of 19-nortestosterone in 0.1 ml. of corn oil, when injected on the 5th day of incubation into the eggs of Wisco-white chickens, completely inhibits the development of the bursa of Fabricius. The hatched chicks are, therefore, bursaless.

In the normal chick the bursal primordium appears as a proliferated epithelial mass in the dorso-caudal region of the embryonal cloaca on the 5th day of incubation. By 168 hours of incubation, it forms a tubular structure communicating with the proctodial canal. Its terminal end enlarges into a vesicle after 216 hours. On the 10th day the bursal vesicle becomes plicated; on the 12th day epithelial buds are given off which become lymphoidal in nature by the 15th day. Thus, the bursa is fully formed and lymphoidal in 17 day old embryos.

There is vacuolization of the bursal primordium in early stages in 19-nortestosterone-treated embryos. It remains as a convoluted short bursal duct without a bursal vesicle. It has been suggested that this may be due to the suppression of mitosis in the bursal primordium.

The secondary effects of 19-nortestosterone on the rectal complex are the stunted growth of the ventral anal lip, increase of musculature in the dorsal lip and attenuation of the proctodial canal with few mucoid glands. These effects are correlated with the accumulation of feces in the cloaca.

Acknowledgment

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THE DISTRIBUTION OF RADIOACTIVITY IN NON-EXCRETORY ORGANS OF THE MALE RAT AFTER INJECTION OF TESTOSTERONE-4-C^{14} [1,2,3]

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ABSTRACT

After the subcutaneous injection of physiologic amounts (about 0.1 mg.) of testosterone-4-C^{14} of high specific activity, significant concentrations of radioactivity were found in several non-excretory tissues of pubescent rats.

The concentrations of radioactivity localized in ventral prostates and other accessory sex glands averaged about two and a half times as great as that in muscle.

The average prostatic concentration of radioactivity in recently castrated animals was not significantly different from that detected in intact pubescent rats, but the levels were more variable from animal to animal in the castrates.

The localization of radioactivity after testosterone-4-C^{14} administration was not influenced by the presence of biliary fistulae.

It is suggested that the radioactivity detected in the tissues probably represented androgen or a derivative.

ENDOCRINE regulation of metabolic processes involves the production, secretion, transport, and utilization of minute amounts of hormonal material. *In vivo*, at the target organ level, the quantity of hormonal substance required to produce profound metabolic and anatomic changes is often so small that it is not detectable by even the most sensitive chemical procedures. Isotope techniques, however, permit detection of such minute quantities of material that physiologic amounts of isotopically labelled hormones might be determinable in tissues.

Previous investigators have been unable to find significant amounts of radioactivity in the accessory sex organs of male animals after the administration of radioactive labelled androgen. Barry, Eidinoff, Dobriner and Gallagher (1) administered testosterone-4-C^{14} intraperitoneally in propylene glycol to rats and mice; but their hormone preparation was of low specific activity (each animal received only 50,000–175,000 disintegrations

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[2] Testosterone-4-C^{14} obtained from Tracerlab, Inc., Waltham, Mass.

[3] Presented at the November, 1957 meeting of the Central Society for Clinical Research and published in the abstracts of that meeting.

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per minute) and significant concentrations of radioactivity were not detected in the sex organs of their animals. Holmes, (2) although primarily interested in the transport of testosterone by plasma proteins, also failed to find significant radioactivity in the sex organs of mature male mice after the administration of testosterone-4-C^{14}.

In the studies to be reported here testosterone-4-C^{14} of high specific activity was dissolved in sesame oil and administered subcutaneously to castrated and intact pubescent male rats in an effort to demonstrate the localization of the isotope in responsive tissues.

METHODS

In all experiments an attempt was made to duplicate physiologic conditions as closely as possible. The dose of testosterone approximated the estimated requirement of 0.1 mg. per day (3) and the steroid was dissolved in sesame oil and injected subcutaneously with the expectation that slow absorption from a depot would simulate the probable continuous delivery of androgen from the testis. Initially, recently castrated pubescent animals were used in order to provide optimal conditions for demonstrating

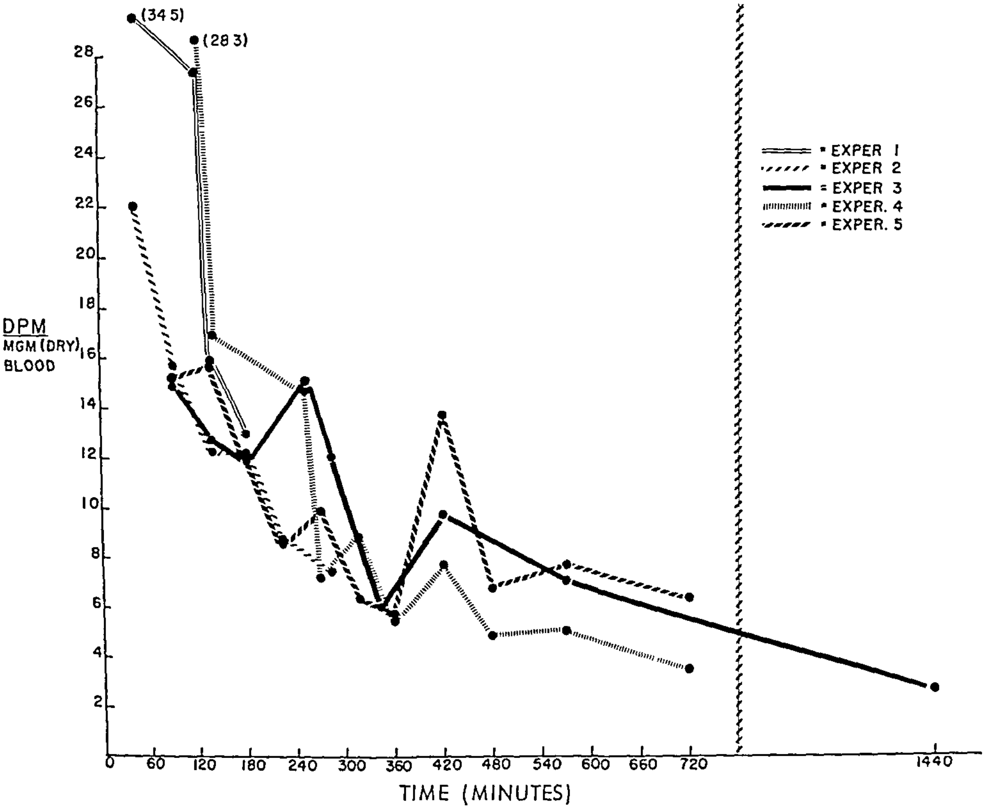

FIG. 1. Radioactivity (disintegrations per minute per mgm. dry weight) in the blood of 45-day old Sprague-Dawley rats which had been given 1.3 microcuries of testosterone-4-C^{14}. Each dot represents the radioactivity in the blood of an animal killed at the time indicated after administration of the isotope. Lines connect the values secured on one experimental day.

androgen localization in accessory sex glands. Hooker (4) showed that pubertal sex accessory tissue is more sensitive to androgen than pre-pubertal or mature glands, and it was felt that this enhanced sensitivity might be associated with an increased avidity of these tissues for testosterone. Castration, by removing the source of endogenous testosterone, would produce a state of androgen need in the partially developed accessory sex tissues.

Testosterone-4-C^{14} with a specific activity of approximately 12 microcuries per milligram was dissolved in sesame oil so that the final stock solution contained 6.5 microcuries per ml. The experiments in which animals were sacrified at various post-injection times (Figs. 1, 2, 3) were done with 45 day old Sprague-Dawley rats which had been castrated three days previously. Each animal was given a 0.2 ml. subcutaneous injection of the stock solution in the dorsal neck region. Care was exercised to avoid leakage of the oily solution from the injection site, and the injected area was vigorously massaged to insure even diffusion of the solution throughout the subcutaneous tissues. The animals were fed Purina chow for several days prior to the experimental period, but were given no food or water after the testosterone injection.

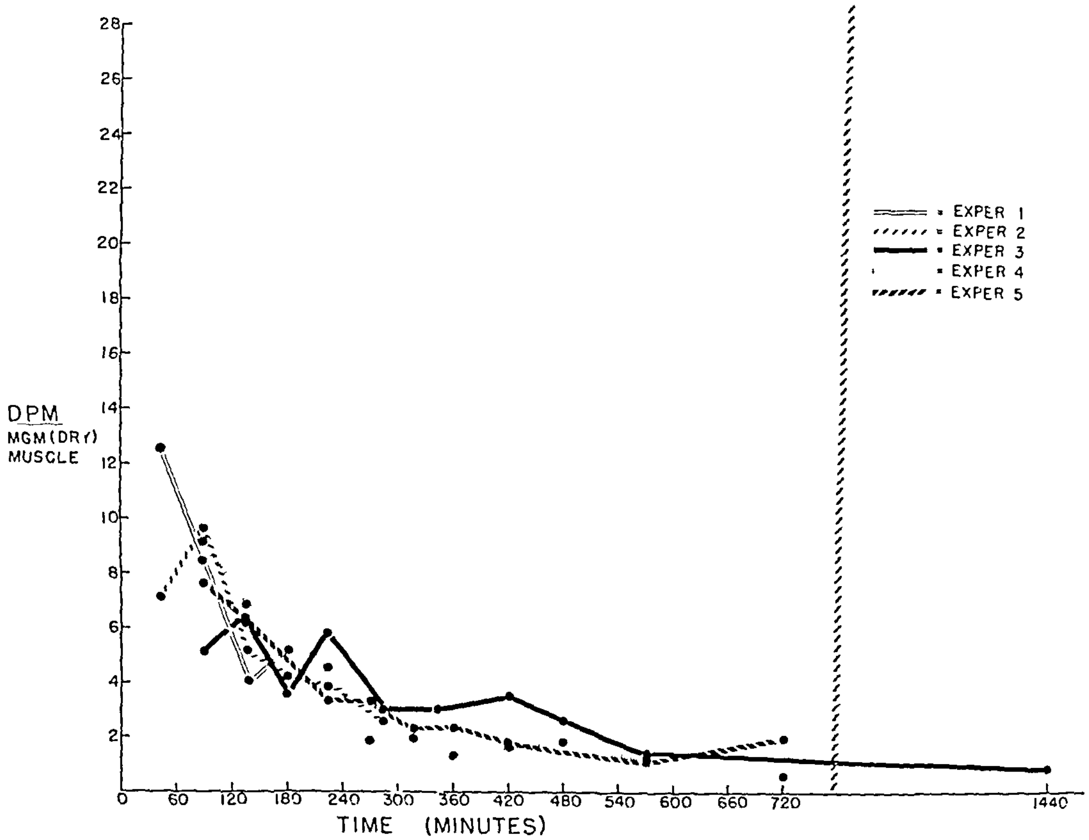

FIG. 2. Radioactivity (disintegrations per minute per mgm. dry weight) in the gastrocnemius of 45-day old Sprague-Dawley rats which had been given 1.3 microcuries of testosterone-4-C^{14}. Each dot represents the radioactivity in the gastrocnemius of an animal killed at the time indicated after administration of the isotope. Lines connect the values secured on one experimental day.

At the time of sacrifice (with ether), a heart blood sample was obtained and then a large bore needle was inserted into the aorta of each animal through the beating left ventricle. The inferior vena cava was cut just above the diaphragm, and the animal was perfused with 100 ml. of normal saline at a pressure of 4–6 feet of water. The sex organs, which invariably appeared bloodless, were then excised under a dissecting lens, blotted, and homogenized in 2.5 ml. of water using glass tissue grinders. Samples of gastrocnemius were homogenized in a similar fashion. Aliquots of each homogenate and of diluted blood specimens (2 ml.) were transferred to weighed copper planchets and evaporated to dryness. The planchets were then reweighed and radioactivity was measured in windowless gas-flow counters. Corrections were made for self-absorption.

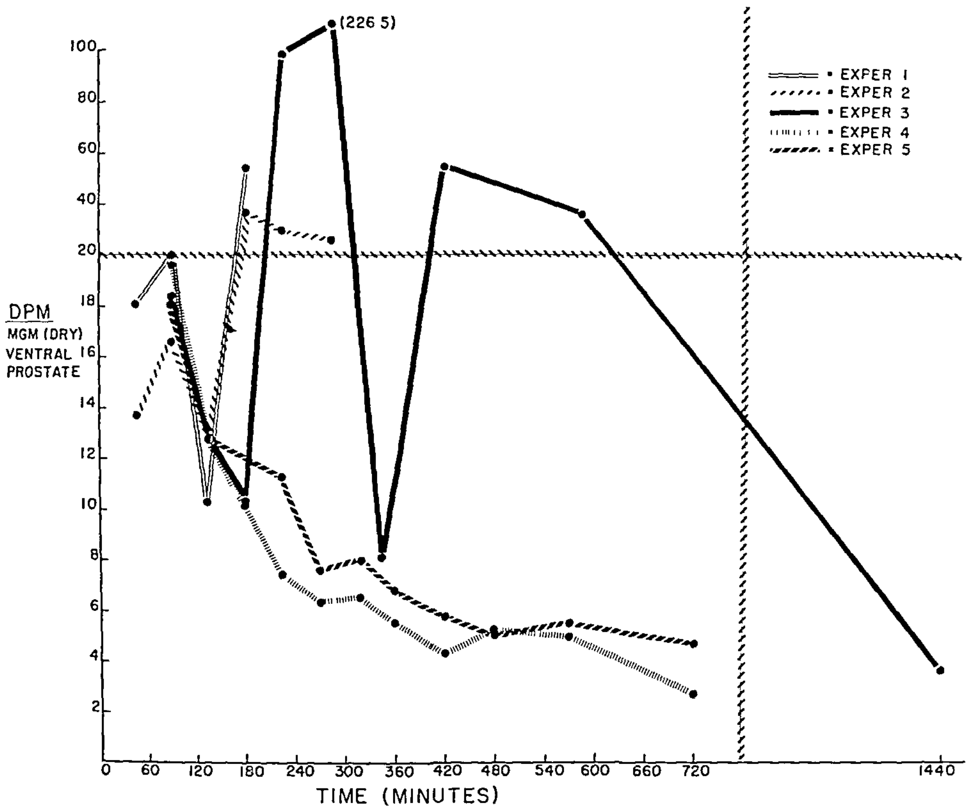

FIG. 3. Radioactivity (disintegrations per minute per mgm. dry weight) in the ventral prostate of 45-day old Sprague-Dawley rats which had been given 1.3 microcuries of testosterone-4-C^{14}. Each dot represents the radioactivity in the ventral prostate of an animal killed at the time indicated after administration of the isotope. Lines connect the values secured on one experimental day.

All values were expressed as disintegrations per minute per milligram dry weight of tissue.

In a second series of experiments in which all animals were sacrificed 300 minutes post-injection, 52-day old male Sprague-Dawley rats weighing approximately 200 gm. were used. These animals were fed a hormone-free diet containing multivitamins, and adequate salt mix, casein, alphocel, dextrin, Mazola oil, oleum percomorpheum and water for several days prior to the experimental period (5). Some of these animals were castrated two days prior to the injection of testosterone-4-C^{14}. Others from the same lot were used intact. Each of these animals received 0.01 ml. of the stock solution per 10 gm. body weight. None of these animals was perfused. The muscle used for comparison was tongue. Tissues were blotted and weighed before homogenization on a Roller-Smith torsion balance. All other procedures used were the same as those noted above except that all values were expressed as disintegrations per minute per 10 mg. wet weight of tissue.

RESULTS

Significant amounts of radioactivity were found in the blood, muscle, accessory sex glands, testis, adrenals, and intestinal walls of pubescent male rats after the administration of C-14 labelled testosterone. In Figure 1 the concentration of radioactivity in the blood of 3-day castrated 45-day old rats is plotted against time. Although there were some irregularities, it can be readily seen that the concentration of isotope dropped rapidly during the first four hours and then more slowly, the biological decay rate approaching an exponential function. Radioactivity was detected 24 hours after administration in one animal. The radioactivity found in gastrocnemius muscle followed a parallel course but was lower throughout (Fig. 2). The isotope concentration in the ventral prostates of these animals also followed the same general course (Fig. 3) but the level was always higher than that observed in the muscle and often greater than that in the blood of the same animals. Since perfusion removed very little of the radioactivity, it may be presumed that the isotope is muscle and prostate was located in extravascular spaces probably bound to the cells.

In occasional animals very high concentrations of radioactivity were found in the prostate. These unexpected results could not be explained by technical or dietary factors, both of which were exhaustively searched for in other experiments. It may be that these exceptional values were related to the age of the rats, for they were never noted in 52-day old animals.

For the purpose of developing a standard procedure which could be utilized for physiologic comparisons, a 300 minute interval following administration of testosterone-4-C^{14} was chosen as the time for sacrificing rats. This time was selected because at 300 minutes the rate of decline in the concentration of radioactivity in the tissues was becoming less rapid, but the isotope concentration was still sufficiently high to permit relatively short counting times. Fifty-two day old rats were used because, as previously mentioned, the uptake of C^{14} by the ventral prostate seemed to vary less from animal to animal at this age.

A new device for presenting isotope concentration data was evolved, the ventral prostate/muscle ratio. This expression tends to cancel the effect of variations in circulating levels of radioactivity and thus makes results obtained at different blood levels more comparable (see below). The tongue, consisting largely of muscle which is not androgen dependent, is then a "baseline" tissue, and the ratio becomes an index of the avidity of the prostate for androgen-derived radioactivity.

Under these conditions the special isotope localizing capability of ventral prostate was clearly brought out (Table 1). With but two exceptions the concentration of isotope in blood was exceeded by that in the ventral prostate. In both the castrated and intact animals the radioactivity in the prostates was with one exception substantially greater than that in tongue muscle. In castrates the prostatic radioactivity averaged 2.87 times as much as muscle; in intact rats the prostate concentrated 2.46 times as much (P of both groups less than 0.001). There was no significant difference between the average ratios in castrate and intact rats, but the values in individual animals were more variable in the castrates.

TABLE 1. THE CONCENTRATION OF RADIOACTIVITY (DPM/10 MG. WET WEIGHT) IN THE BLOOD, VENTRAL PROSTATES AND MUSCLE (TONGUE) OF 52-DAY-OLD RATS SACRIFICED 300 MINUTES AFTER INJECTION OF TESTOSTERONE-4-C^{14}

Castrate animals				Intact animals			
Blood	Ventral prostate	Muscle	VP/M	Blood	Ventral prostate	Muscle	VP/M
7.1	13.1	3.0	4.37	3.2	12.4	3.9	3.18
12.2	21.2	8.1	2.62	3.7	8.8	4.1	2.12
9.1	14.5	6.0	2.42	3.9	10.1	3.3	3.06
4.9	16.1	2.9	5.55	9.8	14.1	10.7	1.32
5.7	17.2	4.8	3.58	3.7	8.7	4.3	2.02
7.5	17.1	6.0	2.85	6.0	10.4	4.8	2.17
10.1	12.4	7.6	1.63	6.6	14.4	5.6	2.52
14.3	16.0	6.0	2.67	6.6	15.0	5.6	2.68
13.4	18.3	6.4	2.86	5.8	13.0	4.6	2.83
4.8	11.9	4.6	2.59	3.0	11.9	4.3	2.77
5.6	14.7	4.8	3.06	6.2	12.6	5.2	2.42
14.0	9.3	10.2	.91				
7.5	12.1	4.6	2.63				
9.5	9.1	5.9	1.54				
13.7	14.1	8.7	1.62				
6.5	14.4	4.4	3.27				
6.0	16.0	5.5	2.91				
7.2	16.8	4.8	3.50				
11.3	16.6	4.5	3.69				
8.9	16.8	4.8	3.50				
6.4	16.2	6.4	2.53				
4.7	14.8	4.7	3.15				
5.1	9.9	3.9	2.54				
Mean 8.5	14.7	5.6	2.87	5.3	11.9	5.1	2.46

The influence of blood levels of the isotope was demonstrated by administering several doses of testosterone-4-C^{14} (Table 2). It can be seen that the level of isotope in blood rose progressively as the dosage of labelled testosterone was increased, and the concentration in the tongue tended to follow that of blood rather closely. The concentration of isotope in ventral prostate, however, did not follow that of blood consistently but was higher than that in blood or muscle at all three dosages.

It would be premature to state at this time that the radioactivity found represented testosterone or a physiologically active androgen derived from it. Preliminary experiments have, however, suggested that the isotope content of the tissues has some direct relationship to the administered androgen. The major route of excretion of radioactivity derived from tes-

TABLE 2. EFFECT OF VARYING THE DOSE OF TESTOSTERONE-4-C^{14} ON THE CONCENTRATION OF RADIOACTIVITY (DPM/10 MG. WET WEIGHT) IN BLOOD, VENTRAL PROSTATES AND MUSCLE (TONGUE) OF 52-DAY-OLD RATS CASTRATED 48 HOURS PRIOR TO INJECTION AND SACRIFICED 300 MINUTES POST-INJECTION

Dose	Blood	Muscle	Ventral prostate	VP/M
.1 cc.	2.2	1.7	5.7	3.35
.2	3.5	2.8	8.0	2.85
.3	4.1	3.7	6.5	1.75
.1	1.7	1.4	5.3	3.78
.2	2.3	2.2	5.7	2.59
.3	4.4	4.0	10.1	2.52
.1	1.2	0.6*	5.1	8.50
.2	2.7	2.3	7.1	3.09
.3	3.3	3.2	5.8	1.81

* Concentration too low for significant count.

tosterone in the rat is via the liver (1). It is therefore of significance that common bile duct cannulation and consequent elimination of liver-excreted radioactivity did not change the absolute concentration of radioactivity in the ventral prostate or the ventral prostate tongue ratio (Table 3). The tissue radioactivity measured thus could not represent hepatic or intestinal degradation products of testosterone-4-C^{14} which had been reabsorbed from the gastroinestinal tract.

The radioactivity in the seminal vesicles was of the same order of magnitude as that found in ventral prostates after the administration of testosterone-4-C^{14}, but the concentrations were more variable from animal to animal. In addition to muscle, salivary glands, adrenals, testes and the colons of bile cannulated animals were sampled, but in none of these tissues was there as high a concentration of the isotope as in the accessory sex glands. The higher levels of androgen derived radioactivity thus seemed to be confined to androgen dependent tissues.

DISCUSSION

The fact that radioactivity was localized in non-excretory tissues after testosterone-4-C^{14} administration in these studies in contrast with the failure to demonstrate such localization by Barry, *et al.* (1) can probably be explained on the basis of the high specific activity of this testosterone. With

TABLE 3. THE CONCENTRATION OF RADIOACTIVITY (DISINTEGRATIONS PER MINUTE PER 10 MGM. WET WEIGHT) IN THE BLOOD, VENTRAL PROSTATES AND MUSCLE (TONGUE) OF RATS WITH CANNULATED BILE DUCTS

	Blood	Prostate	Muscle	VP/M
	—	24.9	4.9	5.08
	12.3	15.7	11.0	1.43
	5.3	13.8	6.0	2.30
	9.4	12.3	5.1	2.41
	1.5	4.7	2.6	1.80
Mean	7.12	14.3	5.9	2.60

the product available, it was possible to inject 2.5–3.0×10^{6} D.P.M. and yet keep the dose of testosterone within the physiologic range of about 0.1 mg. per animal.

Despite the large amount of radioactivity injected tissue levels five hours after injection were in the range of 10–20 D.P.M. per 10 mg. wet weight of tissue. Such a yield may seem insignificant, but dilution in the animal is immense. The ventral prostate comprises less than 0.1% of the total volume of distribution in 52-day old rats. Absorption from sesame oil is slow, and excretion of radioactivity rapid; blood concentrations therefore never achieve very high levels. At five hours post-injection it seems likely, from our experience and that of previous workers (1, 2), that most of the injected radioactivity is in excretory organs. Precise calculation is impossible but these considerations have led us to conclude that yields of radioactivity of the order of magnitude reported in this study were to be expected.

The fact that muscle and other tissues which are not frankly androgen dependent attracted and held some of the radioactive isotope calls for some comment. Testosterone promotes protein anabolism generally (6, 7), and this process might well require the localization in muscle of the androgen or a derivative in a manner similar to that noted in accessory sex tissues. The close correlation between the blood levels of radioactivity and the concentration in muscle suggests that the muscle localization may be a relatively passive phenomenon. The substantially greater concentration in accessory sex glands would accordingly point to the existence of a more active localizing process in androgen-dependent organs. However, it is also possible that this difference may be due to the relative rates at which the tissues involved metabolize the isotope containing compound to another which does not remain in situ. Thus a more rapid dissipation of the androgen or its derivative in muscle would lead to a lower tissue level of isotope than that found in the ventral prostate.

These results do not prove that testosterone is localized in the process of regulating cellular activities. Nevertheless, the fact that those organs which are most dependent upon androgen for their growth and function are the ones which maintain the higher concentrations of isotope after the administration of testosterone-4-C^{14} is suggestive. The identification of the isotope-bearing compound in the tissues is obviously of prime importance, but the significance of these findings would be much the same if the radioactivity represented a metabolite or metabolites of testosterone. The regulation of such a suggested localizing mechanism as well as the controlling of the rate of dissipation of androgen by tissues could be important aspects in the endocrine control of tissue metabolism.

Acknowledgment

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WATER AND ELECTROLYTE CONTENT OF THE UTERUS OF THE INTACT AND ADRENALECTOMIZED RAT TREATED WITH RELAXIN AND VARIOUS STEROID HORMONES[1]

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ABSTRACT

The effect of relaxin[3] administration on the water and electrolyte content of the uterus of the intact and adrenalectomized, immature rat was determined. Uterine water response to the simultaneous administration of estradiol and relaxin was also measured. Relaxin (10 G.P.U.) caused a uterine water increase from a control value of 80.2% to 84.2%. Adrenalectomy caused a 4 to 6-fold increase in sensitivity to relaxin and an increase in the magnitude of the response. Simultaneous administration of minimally effective doses of relaxin and estradiol prevented the uterine water increase while intermediate doses resulted in an additive effect. Progesterone (2 mg.) partially inhibited the action of 10 G.P.U. of relaxin while 1.25 mg. of cortisone acetate completely inhibited the action of 10 G.P.U. of relaxin. The concentration of sodium underwent a slight increase at six hours following relaxin administration but at eighteen had fallen significantly below the normal level. Potassium concentration underwent a slight decrease at six hours, but at eighteen hours had risen above the control level.

IT HAS been known for a number of years that cyclic fluctuations in the water content of the uterus are under hormonal control (1), and that estrogen administration to immature rats causes an increased uterine water content, comparable to that observed in late proestrus (2). It has been shown recently that relaxin administration to rats produces an increase in uterine water content, when given alone (3), or following pretreatment with estrogen (4).

The present report is concerned with the relationship of relaxin to the steroid hormones in its action on the water and electrolyte content of the uterus in the intact and adrenalectomized rat. Specific attention was paid to the influence of estradiol, progesterone, and cortisone on the action of

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[3] Relaxin was supplied under the trade name of Releasin (Lot No. 004093) through the courtesy of Dr. Robert L. Kroc, Warner-Chilcott Laboratories, Morris Plains, New Jersey.

relaxin. Sodium and potassium concentrations in the uterus were determined at various intervals following the administration of relaxin, in an effort to elucidate the mechanism by which uterine imbibition occurs.

MATERIALS AND METHODS

Immature rats of the Holtzman or Purdue strain were used throughout these studies. The animals were weaned at 21 days of age, and placed upon a liquid diet (10% glucose in the drinking water) for three days. This was found necessary in order to insure a consistent uterine water content of approximately 80% in the untreated animals. Earlier studies had revealed abnormally high concentrations of water in normal uteri, which could be explained by the presence of estrogen in the food (5). In determining the role of the adrenal cortex on this phenomenon, animals were bilaterally adrenalectomized on the day of weaning, and placed on a liquid diet, (10% glucose and 1% sodium chloride in the drinking water). In addition, each animal received a single subcutaneous injection of 500 μg. desoxycorticosterone acetate immediately following adrenalectomy.

Estradiol was dissolved in sesame oil and injected in doses ranging from 0.005 μg. to 0.1 μg. Relaxin solutions were prepared from a stock solution of Releasin,[3] and administered in doses ranging from 0.05 G.P.U. to 100 G.P.U. All injections were made subcutaneously, and each injection was given in a volume of 0.1 ml. In previous experiments (2, 3), it was demonstrated that maximum uterine water concentration following a single injection of either 0.1 μg. estradiol or 50 G.P.U. relaxin occurs six hours after administration. Accordingly all treated animals were killed six hours following injection.

The animals were killed by ether, weighed, and the uterus quickly removed from the body cavity. The uterus was dissected free of fat, and a longitudinal incision made in the antimesometrial surface, extending from the ovarian end to the cervical junction, in order to allow the escape of lumenal water. The uterus was then gently rolled on bibulous paper to remove water adhering to the outer surface, and weighed to the nearest 0.1 mg. on a torsion balance. The uteri were dried in a vacuum oven at 50° C, weighed again, and the water content calculated.

Dried uterine tissue was wet ashed in a 2 ml. mixture containing five parts concentrated nitric acid to one part perchloric acid. This mixture was then dry ashed at 550° C for 12 hours. The ashed residue was brought to 10 ml. volume with triple distilled water, and analyzed for sodium and potassium content in the flame photometer. The "direct intensity" method was used for determination of both ions, since interfering ions were absent in the concentrations used.

RESULTS

The lowest dose of relaxin which increased significantly the per cent of water in the uterus of the intact rat was 1 G.P.U. (Fig. 1). This dose of relaxin increased the per cent uterine water from a control value of 80.2% to a value of 81.4%. Although an effect was obtained following the administration of 0.1 G.P.U. statistical analysis by the Newman-Keuls sequential range test demonstrated no significant difference at the 1% level. A maximum response (84.2% water) was obtained following the injection of 10 G.P.U. relaxin. Further increase in the dosage of relaxin to 50 and 100 G.P.U. failed to elicit a significantly greater response.

The administration of progesterone partially inhibited the increase in

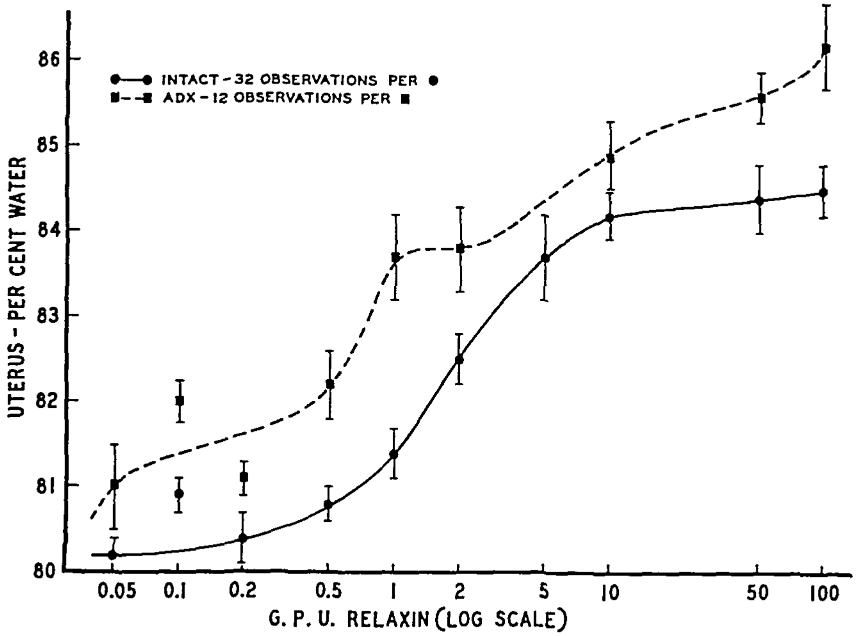

FIG. 1. Increase in the per cent uterine water in the immature rat treated with relaxin.

●—intact rat
■—adrenalectomized rat

uterine water content observed following administration of relaxin alone (Table 1). Treatment with 10 G.P.U. of relaxin and 2 mg. of progesterone increased the uterine water to 81.7% as compared with 84.2% for relaxin alone. Lower dosages of progesterone with the relaxin held constant at 10 G.P.U. produced a correspondingly smaller degree of inhibition. Doses of 0.25 and 0.5 mg. progesterone failed to induce any inhibition of the action of 10 G.P.U. of relaxin on the uterine water content, while 1 mg. of progesterone caused a 41% inhibition and 2 mg. progesterone caused a 61% inhibition.

Adrenalectomy increased the uterine water response to relaxin. Although

TABLE 1. UTERINE WATER CONTENT OF THE 24-DAY-OLD RAT AT 6 HOURS FOLLOWING THE SIMULTANEOUS ADMINISTRATION OF RELAXIN AND PROGESTERONE

No. of animals	Relaxin G.P.U.	Progesterone, mg.	Uterine water—%
10	—	—	80.1 ±0.22*
10	10	—	84.2 ±0.32
10	10	0.25	83.9 ±0.42
10	10	0.5	84.3 ±0.38
10	10	1.0	82.5 ±0.26
10	10	2 0	81.7 ±0.30

* Standard error of the mean.

the slope of the curve for the adrenalectomized rat is not similar to that obtained for the normal rat it is possible to estimate an increased sensitivity of the order of 4 to 6 fold (Fig. 1). A significant increase in uterine water (82.0%) was obtained with 0.1 G.P.U. of relaxin and a maximum effect (86.2%) was obtained following 100 G.P.U. of relaxin. Treatment with cortisone acetate inhibited the response of the adrenalectomized rat uterus to relaxin. A 44% inhibition was obtained with 0.5 mg. cortisone acetate in rats injected with 10 G.P.U. of relaxin and complete inhibition was obtained with 1.25 mg. cortisone acetate (Table 2).

TABLE 2. UTERINE WATER CONTENT OF THE ADRENALECTOMIZED 24-DAY-OLD RAT 6 HOURS FOLLOWING THE SIMULTANEOUS ADMINISTRATION OF RELAXIN AND CORTISONE ACETATE

No. of animals	Relaxin, G.P.U.	Cortisone acetate, mg.	Uterine water—%
10	—	—	80.1 ±0.58*
10	10	—	84.9 ±0.46
10	10	0.5	82.8 ±0.56
10	10	1.25	80.0 ±0.50
10	10	2.5	80.9 ±0.42
10	10	10.0	80.0 ±0.40

* *Standard error of the mean.*

The simultaneous administration of relaxin and estradiol produced varied responses with regard to the uterine water depending on the dosage of each hormone (Fig. 2). The simultaneous administration of 0.005 μg. estradiol and either 0.2 or 0.5 G.P.U. relaxin inhibited the response seen with relaxin alone at that level.

Increasing the dosage of relaxin to 1 G.P.U. caused an additive effect, while further increases in dosage to 2 and 10 G.P.U. produced no greater degree of water imbibition than that observed with relaxin alone. When the level of estradiol was raised to 0.01 μg., the antagonism between the two hormones was apparent only at the lowest dosage of relaxin administered. Each dose above this minimally effective level of relaxin caused an additive result, while the two high relaxin dosages actually produced a synergistic effect on the uterine water response. When 0.02 μg. estradiol was administered, no antagonism was present at any of the concomitantly administered relaxin doses, and in fact, a significant additive effect occurred with 1 and 2 G.P.U. relaxin.

Concentrations of sodium and potassium in the immature rat uterus at definite time intervals following the separate administration of maximally effective doses of relaxin and estradiol are illustrated in Table 3. Following the administration of 0.1 μg. estradiol a rise in sodium from the control level of 59.5 m.eq. to 74.0 m.eq. and a decrease in potassium from 62.0 m.eq. to 40.0 m.eq. occurred at 6 hours. Thirty hours following hormone injection, the sodium level returned to normal, and the potassium con-

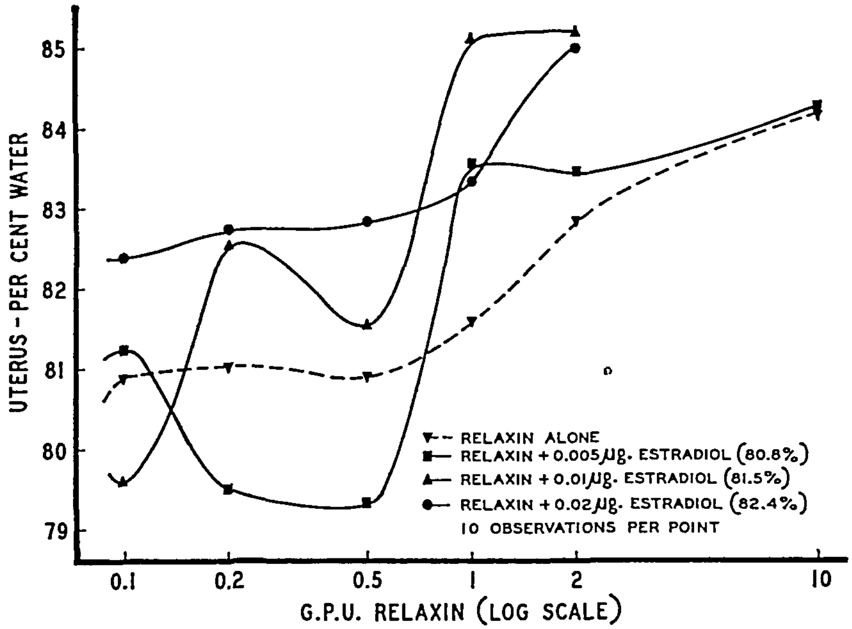

FIG. 2. The effect of simultaneous administration of estradiol and relaxin on the per cent uterine water in the immature rat. Note: Uterine water responses to the three levels of estradiol, when administered alone, are indicated in parentheses.

centration returned to a point approaching the normal level. Six hours after the administration of 50 G.P.U. relaxin there was a rise in sodium to 67.5 m.eq., and at 18 hours a drop to 46.5 m.eq. had occurred. This was accompanied by an increase in potassium from 55.0 m.eq. at 6 hours to

TABLE 3. THE EFFECT OF RELAXIN AND ESTRADIOL ON THE CONCENTRATION OF SODIUM AND POTASSIUM IN THE UTERUS OF THE 24-DAY-OLD RAT

Treatment	Time post injection	No. of animals	Uterine water %	m.eq. Na/Kg. wet wgt.*	% change**	m.eq. K/KG. wet wgt.*	% change**
None	0	5	80.2	59.5	—	62.0	—
Relaxin (50 G.P.U.)	6	5	84.4	67.5	+13**	55.0	−11
Relaxin (50 G.P.U.)	18	5	82.0	46.5	+22**	101.0	+63**
Relaxin (50 G.P.U.)	30	5	79.6	61.5	+ 3	51.0	−21**
Estradiol (0.1μg)	6	5	86.0	74.0	+25**	40.0	−35**
Estradiol (0.1 μg)	30	5	82.2	61.5	+ 3	55.0	−11
None (ADX)	0	5	80.1	54.0	− 8**	110.0	+75**
Relaxin (50 G.P.U.-ADX)	6	5	85.5	49.0	− 8**	90.0	−18**

$$* \text{ m.eq./Kg. Wet Wgt.} = \frac{\dfrac{\text{m.eq./L.}}{100}}{\text{Wet Wgt.}} \times 10^6.$$

** Indicates significant difference from control value as determined by the Student "t" test at the 1% level of significance. Electrolyte concentrations of the adrenalectomized animals which received relaxin were compared with those of the adrenalectomized controls All others were compared with electrolyte concentrations of the intact controls.

101.0 m.eq. at 18 hours. As is the case with estradiol, both sodium and potassium levels had returned to points approaching normal, 30 hours after relaxin administration. In adrenalectomized animals the potassium level was significantly higher than in the intact controls, while the sodium concentration was slightly lower. Both underwent a decrease 6 hours following the administration of relaxin.

DISCUSSION

The present results indicate a similarity in action of the two hormones, estradiol and relaxin, in their ability to cause an increased uterine water content in the rat. These data confirm the finding that relaxin can produce this effect without the necessity of previous priming with estrogen (3). In a series of other effects produced by relaxin, such as separation of the pubic symphysis of the guinea pig and mouse (6, 7), dilation of the uterine cervix in the cow and sow (8, 9), and inhibition of uterine contractions in the rat (10, 11), it was necessary to first sensitize the responsive tissue with estrogen.

The role of the adrenal cortex in this phenomenon has heretofore been rather neglected. Szego and Roberts, (12), in studying pituitary-adrenal cortical relationships to the uterine water phenomenon, found that adrenalectomy significantly enhanced the response to estrogen. Our results have indicated a similar role of the adrenal cortex in reducing the uterine water response to relaxin administration. The sensitivity of the uterus to relaxin is increased by a factor of 4 to 6 following adrenalectomy. The minimal effective dose of the hormone is decreased and the maximum response is increased. Further evidence in support of an effect by the adrenal cortex in this phenomenon is illustrated by the finding that administration of 0.5 mg. cortisone acetate partially inhibits the uterine water increase observed following 10 G.P.U. relaxin, while 1.25 mg. cortisone acetate completely blocks the response. Progesterone also produced a partial inhibition of the action of relaxin on the uterine water. In this respect both cortisone and progesterone had the same action in preventing or partially inhibiting the increased uterine water content following relaxin or estradiol (12).

The mechanism of action of both estradiol and relaxin in causing the uterine water increase is at present not clear, but the depressant action of the adrenal cortex would appear to suggest that the electrolytes may be involved in this phenomenon. Talbot et al. (13) correlated a rise in sodium concentration with the increased uterine water content, at 6 hours following estrogen administration and demonstrated a rise in potassium content at 30 hours, which was the time at which a secondary rise in percent water and dry weight of the uterus occurred. Our findings indicate that the electrolyte pattern following relaxin administration generally follows the picture observed following estrogen.

There appears to be no doubt that a similarity in action exists between estradiol and relaxin. Furthermore, an actual potentiating action of relaxin with estrogen has been noted in the rat (14), and in the mouse (15). The present results indicate that the simultaneous administration of estradiol and relaxin may lead to an inhibition of the effect seen with relaxin alone or to a synergism. While minimally effective doses of estradiol and relaxin cause a depression of uterine water content, other dosages show either an additive effect or no effect, different from that observed following either hormone, when administered alone. There appears to be no definite ratio of these hormones at which synergism results, but the data definitely indicate a true complemental action.

It is obvious that the relationship of the two hormones in their action on uterine water content of the rat is not a simple one.

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ENDOGENOUS AND EXOGENOUS VASOPRESSIN ON ACTH RELEASE[1]

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ABSTRACT

Simultaneous studies of the release of antidiuretic hormone (vasopressin, ADH) and corticotrophin (ACTH) were performed in unanesthetized trained dogs. Antidiuresis was used as the index of ADH liberation and plasma free 17-hydroxy-corticosteroid levels were used to indicate ACTH release. No correlation was observed between discharge of ACTH and release or inhibition of the release of ADH. Further studies with highly purified vasopressin showed that exogenous ADH was unable to stimulate the secretion of ACTH until a threshold of $3-7\times10^3$ times the dose reproducing maximal physiologic antidiuresis was exceeded. The physiological significance of the data evidencing ACTH release upon injection of vasopression is debated and questioned.

VASOPRESSIN (ADH) has been proposed as the ultimate mediator of ACTH release on the grounds of several arguments based on inferential conclusions (1) and on the observation that injection of large quantities of the pressor antidiuretic fraction of the posterior lobe of the pituitary stimulates release of ACTH (2, 3, 4, 5, 6). In accordance with this hypothesis, one would expect that acute release of endogenous ADH should associate with observable signs of increased ACTH secretion. Were this not the case, a strong argument would have been procured against the vasopressin theory. To study the validity of this assumption, ADH release as ascertained by the degree of ensuing antidiuresis was induced by intracarotid injections of hypertonic saline in conscious trained dogs and pituitary-adrenal function was explored simultaneously by measurement of plasma free 17-hydroxy-corticosteroid levels. The minimal amount of highly purified vasopressin that would produce ACTH release upon injection in the carotid was also determined and related to amounts of endogenous vasopressin referable to physiological antidiuresis.

MATERIAL AND METHODS

Preparation and care of the animals. Three female dogs weighing 14, 15 and 18 kg. were prepared with a carotid loop and trained for intracarotid injections and measurement of diuresis in the conscious state according to the methods of Verney (7).

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Measurement of diuresis and stimulation of antidiuresis. The technique chosen for hydration was that of van Dyke (8) which requires two successive water loads by stomach tube. During the second load, urine was collected by means of indwelling catheters at 5 minute intervals; the volume was recorded as average flow per minute per period of collection. Endogenous ADH release was obtained by intra-carotid injection of hypertonic saline (4 to 8%) prepared and administered according to Verney (7) i.e. total volume of 2.0 ml. injected over 5–20 seconds. The per-cent-change of urine flow from the pre-injection level to the lowest flow observed after injection (% antidiuresis) was used as a convenient quantitative expression of the antidiuresis obtained. Lysine-vasopressin (LVP) was prepared by the method of Ward and Guillemin (9) and further purified by electrophoresis. The doses of LVP injected ranged from 0.066 to 2.12 units. These were injected in 2 ml. of 0.9% saline into the carotid artery over a period of 60 seconds. The specific activity of the preparation used was 260 u./mg. when assayed against the USP Posterior Lobe Reference Standard in the rat treated with dibenamine (26).

Assessment of ACTH release. ACTH release was assessed by serial measurements of the plasma free 17-hydroxy-corticosteroid (17OHC) levels. 17OHC were determined by the method of Nelson and Samuels (10) on 20 ml. blood samples. Blood samples were taken from the carotid artery immediately before and 15 and 60 minutes after the injection of hypertonic saline or LVP. The experiments were planned so that no more than 120 ml. of blood were drawn within a single week from each animal. All samples were collected between 11:30 A.M. and 2:00 P.M. With this method, our range for normal (resting) concentrations of 17OHC is 0 to 8 μg./100 ml. of plasma. These values are based on approximately 150 single determinations; they are in agreement with what has been reported in the literature using the same method and on plasma samples obtained at similar times of the diurnal rhythm (30).

RESULTS

Endogenous ADH

a. *Inhibition of release.* (Fig. 1). During the training procedure, a number of blood samples were drawn from the dogs in various stages of the period of hydration and diuresis. When these 17OHC concentrations and the values at Time zero (i.e. at time of maximal diuresis) in subsequent experiments were plotted against the urine flow per minute, no correlation between the variants was evident. Inhibition of the release of ADH can thus be obtained simultaneously with evidence of stimulation of the release of ACTH.

b. *Stimulation of release.* (Table 1). The amounts of hypertonic saline administered were limited by the intrinsic irritant effects of the hypertonic solution for each dog, as some discomfort was observed in most instances (7). This was manifested by smacking of the lips, whining, stretching of the extremities and occasional mild struggling. In 10 experiments the antidiuresis so obtained varied from moderate to maximal. In 8 of these experiments no change of the corticoid levels was observed over the 60 minutes of study, irrespective of the antidiuresis produced. In 2 of these experiments, increased plasma 17OHC levels were observed; in both of these, technical errors had provoked extremely painful reactions of the animals. In one of these experiments, most of a 2 ml. dose of 8% saline was ex-

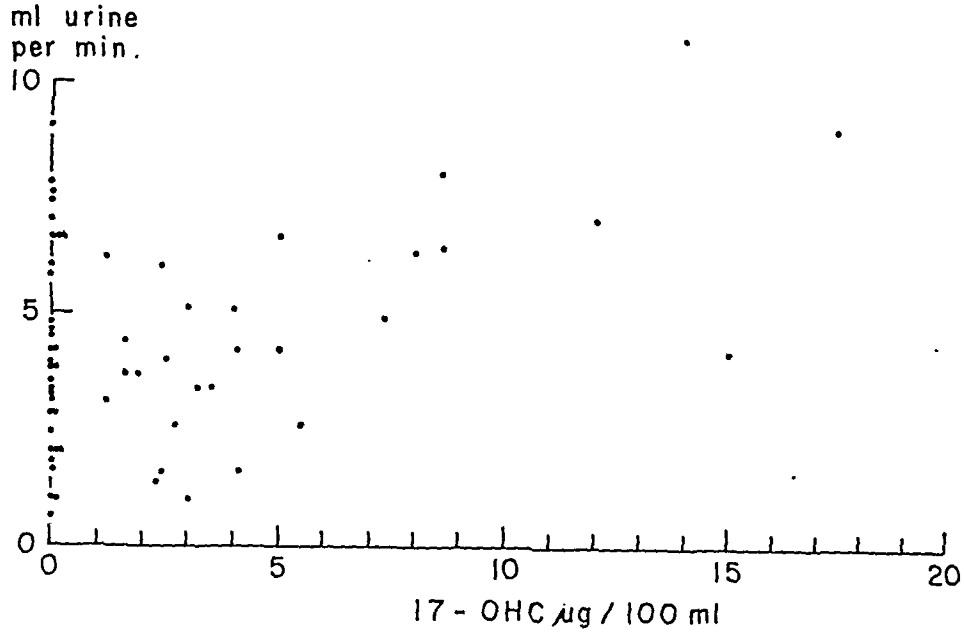

FIG. 1. Scattergram of 17OHC related to urine flow.

travasated; there was only a minimal antidiuretic response. In the other, the 2 ml. of 8% saline were injected within 5 seconds. the dog leaped off the table and howled in obvious reaction to acute pain.

Exogenous ADH. (Table 2). The injection of LVP was always followed by maximal antidiuresis, blanching of the gums, and marked bradycardia. A very distinct rise in the 17OHC levels was observed with the larger doses. A threshold for ACTH release was found in each dog. In 2 animals it was

TABLE 1. ENDOGENOUS ADH. INJECTION OF HYPERTONIC SALINE IN THE CAROTID ARTERY. MEASUREMENT OF ANTIDIURESIS AND LEVELS OF PLASMA 17OHC (TEN TRIALS IN 3 DOGS)

Time of sampling	Plasma 17OHC concentrations in μg./100 ml.									
0	0	0	8.6	0	8.6	0	0	0	0	8.0
15 minutes	0	0	8.2	0	7.3	—	—	0	0.2	16.4
60 minutes	0	0	7.3	0	—	0	0	0	16.7	14.5
% Antidiuresis	48	51	34	69	100	78	81	88	100	21

TABLE 2. EXOGENOUS ADH. INJECTION OF LYSINE-VASOPRESSIN IN THE CAROTID ARTERY. MEASUREMENT OF ANTIDIURESIS AND LEVELS OF PLASMA 17OHC (13 TRIALS IN 3 DOGS)

Time of sampling	Plasma 17OHC concentrations in μg./100 ml.												
0	4.0	0	0	0	0	0	2.5	4.1	2.6	1.9	0	0	0
15 minutes	2.5	0	0	2.1	0	0	5.4	5.6	3.7	4.6	5.5	5.8	2.0
60 minutes	0	0	0	6.0	0	0	2.5	4.1	8.2	0	4.7	3.2	6.2
Doses of LVP in USP Units	.066	.066	.066	.130	.130	.130	.260	.260	.260	.520	.520	1.4	2.12
% Antidiuresis	100	100	100	100	100	100	100	100	100	100	100	100	100

0.260 u. while in the other, stimulation of ACTH release was obtained with 0.130 u. The antidiuresis was so profound in each experiment that comparison of the antidiuretic effects of the different doses of LVP was difficult to assess. In those experiments where a recovery of diuresis was evident before exhaustion of the water load, the time between the onset of antidiuresis and a 50% recovery was calculated. These figures indicated a quantitative relationship between dose and duration of antidiuresis but no correlation with the pituitary-adrenal stimulation. The stimulation of ACTH release appeared as an all-or-none type of response within the range of doses of LVP administered. There was no relation between the dose of LVP and the 15 or 60 minute response once the threshold of stimulation of ACTH release had been exceeded. The most common response consisted of a peak at 15 minutes, followed by a fall at 60 minutes. In three experiments the 15 minute peak was not observed but an almost linear climb to a high 60 minute response was observed. The pattern of the response was not related to the dose of vasopressin administered.

DISCUSSION

It might be argued that a lack of correlation between degrees of antidiuresis and levels of plasma 17OHC in the dog, a species where these are usually very low, is only due to a large difference in sensitivity of the two phenomena to their respective affectors.

The data available in this paper do not provide a quantitative answer to this question. However, numerous observations of increased levels of 17OHC upon stimulation of *endogenous* ACTH release have been obtained in this series of experiments. (Fig. 1, Tables 1 and 2). It is interesting that these increases in plasma 17OHC levels bore no relationship to the degree of antidiuresis observed concomitantly: some of the highest levels of 17OHC observed were found in the maximal *diuresis* of the water loading (Fig. 1) during the training period of the animals when it was an obviously "stressful" procedure to them.

Jewell and Verney have reported (11) that the endogenous release of ADH to produce maximal physiological antidiuresis is in the range of 12×10^8 molecules/second. Knowing the specific activity of the lysine-vasopressin used in these experiments at 260 units/mg., the molecules/second administered can be calculated. Thus the threshold for ACTH stimulation is 4.2×10^{12} to 8.6×10^{12} molecules/second. This indicates a factor of 3.5×10^3 to 7.1×10^3 between the amount necessary for maximal antidiuresis and the threshold dose for ACTH stimulation. If only one-thousandth (1×10^{-3}) of the dose injected into the carotid artery arrived at the receptor site for ACTH release, the above discrepancy could perhaps be explained on the basis of dilution alone. However, by the same reasoning, the endogenous ADH would have to reach this level to stimulate ACTH release. This would dictate a maximal antidiuresis each time ACTH is released. This is not supported in this series of experiments by the high levels of 17OHC found during *diuresis* (Fig. 1) or by this observation of a rise in 17OHC concentration with only minimal antidiuresis (Table 1).

The administration of pressor extracts of the posterior lobe of the pituitary (Pitressin, Protopituitrin) or of highly purified vasopressins of natural or synthetic origin has been reported to produce stimulation of the pituitary-adrenal system (2, 3, 4, 5, 6). If this would appear of questionable meaning in the normal subject (3, 4, 6), the fact observed in preparations in which stress induced ACTH release was impaired or prevented (2, 5, 12) has been taken as strong support of the theory proposing ADH as the physiological mediator of ACTH release. In reaction with this conclusion, it was pointed out (21, 25, 27) that the doses of vasopressin used in the experiments of these various authors are so large that they are of little significance to any mechanism underlying physiological phenomena. Indeed, explanations other than the above apparently obvious conclusions have begun to appear regarding the mechanism of action of these pharmacological doses of vasopressin (13, 14, 15). The results reported here have confirmed that highly purified vasopressin can produce pituitary-adrenal stimulation. This was done by injecting in the carotid doses of the material greatly in excess of those reproducing physiological antidiuresis. Conversely, controlled release of endogenous ADH was never followed by evidence of ACTH release whereas definite signs of pituitary adrenal stimulation were obtained in conditions corresponding to no stimulation or even inhibition of ADH release (Fig. 1). A similar conclusion was recently reached by McDonald (16) in contradistinction to his previous position (3, 4). Only a highly speculative theory could reconcile these data and the postulated role of vasopressin as the ultimate mediator of ACTH release. It would involve a differential release of vasopressin from the posterior lobe, to the periphery for antidiuresis, or to the anterior pituitary for ACTH secretion (by way of the short vessels which have been described recently (17, 18, 19) or by the hypothalamo-hypophysial portal vessels). This hypothesis has indeed been ventured by McCann in private discussions. The physiological complications inherent to this concept are rendered even more unnecessary by the recent demonstration (20, 29) that stimulation of ACTH release can be obtained with a linear dose-response relationship by *peripheral* administration of crude CRF preparations (Guillemin's fraction D), whereas equipressor doses of purified lysine vasopressin given by the same route are completely ineffective in stimulating release of ACTH. Stimulation of the discharge of ACTH by *endogenous* vasopressin may still be possible in these unusual stresses such as massive sudden blood loss where unusually large amounts of ADH appear to be released suddenly (22). Stimulation of the discharge of ACTH by *exogenous* vasopressin will always be possible if large enough doses of the material are administered. Since this is true *in vitro* (23, 24) as well as *in vivo* in animals with hypothalamic lesion or blockade (5, 13, 20, 29), it may be best explained as previously proposed (14, 23) by an overlapping of activities of molecules closely related structurally as seems to be probable for CRF and vasopressin.

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DIURNAL VARIATION IN RESPONSE TO GONADOTROPIN IN THE MOUSE

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ABSTRACT

With the use of immature mice 18–20 days of age it was shown that a single injection of 0.1–10.0 I.U. chorionic gonadotropin (HCG) or pregnant mare serum gonadotropin (PMS) resulted in greater uterine and ovarian weights 24 hours later if given in the afternoon as compared with the morning. A diurnal difference in response was also observed in ovarian but not in uterine weights at 48 hours after injection. The diurnal variation in response was not apparent in mice aged 14–17 days, but was significant in 6 week old mice. In the latter the period of increased responsiveness was more restricted than in mice aged 20–22 days.

A study of the time-response relationships indicated that the greater response in organ weights at 24 hours was a consequence of an earlier beginning of growth rather than an increased rate of growth. Hypophysectomy and high doses of PMS and HCG (30 I.U.) prevented the diurnal variation in response, which was also not apparent in the uterine weight response to a single injection of either oestradiol, post-menopausal urine gonadotropin or a crude extract of anterior pituitary glands.

It is concluded that there is a diurnal variation in pituitary function and not in ovarian or uterine function. The results are discussed in relation to the gonadotropic hormones.

IN STUDIES in this laboratory on the mode of action of various gonadotropin preparations on the mouse ovary, the two responses examined have been ovarian weight and uterine weight. Previous work (12, 14), employed a single injection technique in which the gonadotropin was administered subcutaneously in isotonic saline 44–46 hours before killing.

Before this work could be extended to multiple injection techniques, particularly spread over one day, it was thought important to investigate the possibility of diurnal variation in response to a single injection. This was prompted by the relationship known to exist between oestrus and ovulation and the diurnal rhythm of light and dark in mice and rats (1, 3, 9, 17). Braden (2) has extended this work to show that the time of ovulation

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in mice is affected by changes in the relative length of the light and dark phases of the 24 hour cycle. In studies on the release of the ovulating hormone in rats, Everett and Sawyer (6, 7) have presented evidence which points to the presence of a diurnal rhythm in the excitability of the neural component of the "LH-release apparatus." They have suggested that between 2 and 4 P.M. each day there was in their rats a characteristic excitability of the neural component of the ovulating mechanism and that the particular night during which oestrus and ovulation took place was determined by the interaction of this diurnal pattern and the oestrogen and progesterone produced by the developing follicles.

Preliminary work indicated that there is a diurnal rhythm in the sensitivity of the immature mouse to gonadotropin. The experimental approach was, firstly, to establish clearly this fact, and secondly, to determine the extent of the period of increased sensitivity. In addition, experiments were carried out to compare the time-response relationships in both uterine and ovarian weights to morning and afternoon injections, and to gain some indication of the mechanism of the diurnal rhythm.

MATERIALS

Animals. Immature female albino mice were weaned when 18–20 days old, except where otherwise indicated (Experiment IV). Injections commenced as soon as practicable after weaning. Large groups of mice of similar age were available for each experiment; the method of breeding has been previously described (4). In all experiments the mice were allotted to experimental groups entirely at random. They were housed five mice per cage, each cage containing mice on a particular treatment. The conditions in the animal house were held constant at 78 ± 2° F, 14 air changes per hour, and light: dark ratio of 12:12 hours (lights, fluorescent white, on 6 A.M.–6 P.M.).

All injections of gonadotropins, dissolved in isotonic saline, were made subcutaneously in the dorsal region. The injection volume was 0.1 ml. The mice were killed by cervical dislocation and the body weights recorded immediately. The uteri and ovaries were dissected free of extraneous tissue under a binocular dissecting microscope and weighed on a torsion balance after pressing on filter paper. The uteri were weighed to the nearest 0.5 mg. and the ovaries to the nearest 0.25 mg.

Gonadotropins. The gonadotropic substances used in the majority of experiments were human chorionic gonadotropin (HCG)[3] and pregnant mare serum gonadotropin (PMS).[2] A large number of vials of the same batch of each substance was available.

EXPERIMENTS AND RESULTS

Experiment I

The experimental design was a 2×4×2×3 plus a 2×4×2×2 factorial. In the first, two gonadotropins, each at four dosage levels, were given in a single injection at either 9 A.M. or 9 P.M. This experiment was performed on three successive days. The second experiment, carried out concurrently with the first, was the examination of the effect of storage of gonadotropin over the period of the test. The total number of treatment combinations was 48 plus 32, of which 16 were common to both experiments. Since each group comprised five mice, a total of 320 mice were involved in the two experiments (Table 1). The mice were killed 24 hours after injection.

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[3] Pregnyl and Gestyl obtained from Organon Laboratories Ltd., London.

An important consideration was the possible bias due to time from weaning to infection. This was overcome by allotting mice at random to each of the six times of injection (2 times per day for 3 days) prior to commencement of the experiment. Each batch of mice was then weaned twelve hours before the appropriate time.

The dosage levels of gonadotropin were 0.22, 0.66, 6.6 and 20 nominal I.U. HCG and 0.11, 0.33, 3.3 and 10 nominal I.U. PMS. The two lowest levels of each substance were expected to give responses which fell on the linear portion of the log dose-log uterine response curves (4) and the two highest levels, responses which were on the linear portion of the log dose-ovarian weight response curves (12).

TABLE 1. SCHEDULE OF OPERATIONS IN EXPERIMENT 1

		Mice weaned	Mice injected	Mice killed
Day 0	9 P.M.	40		
Day 1	9 A.M.	40	40	
	9 P.M.	40	40	
Day 2	9 A.M.	40	40	40
	9 A.M.	80	40	40
Day 3	9 A.M.	80	80*	40
	9 P.M.		80*	40
Day 4	9 A.M.			80
	9 P.M.			80

* 40 mice were injected with fresh gonadotropin and 40 were injected with the preparations given on Day 1 and stored for 48 hours.

Immediately prior to injection a vial of gonadotropin was dissolved in the appropriate amount of isotonic saline to give a solution containing the highest dosage level in 0.1 ml. The lower levels were prepared by dilution. The sixteen solutions (two gonadotropins × four levels × two times of the day) employed on the first day were stored in the refrigerator and used again 48 hours later.

The uterine and ovarian data were analysed separately but the statistical methods were similar. The regressions of response on body weight were calculated for eight groups of 20 mice; i.e., the six groups that received the fresh preparation over the three days and the two groups that received the stored preparations on Day 3. The regression coefficients were examined for evidence of heterogeneity, $\chi^2_{(7)} = 13.62$, $0.05 < P < 0.1$ (uterine weights) and $\chi^2_{(7)} = 9.91$, $0.1 < P < 0.2$ (ovarian weights). These results indicated that an overall regression could be used. Hence the group totals were corrected for concomitant variation due to body weight and the corrected totals were used in the analysis.

The value of the regression of log uterine response on log body weight, $b = 1.12$, and the value of the corrected within-group variance, $EMS_c = 71.3$, were similar to previous results (4). In addition the value for the regression of ovarian response on body weight, $b = 0.32$, and the corrected within-group variance, $EMS_c = 0.37$, are typical of results in this laboratory.

The data were analysed in two steps.

(i) Comparisons were made between preparations, doses, times of day and replications using the fresh preparations.

(ii) Comparisons were made between preparations, doses, times of day and stored versus fresh materials for the results of the injections carried out on Day 3.

The analyses of variance are presented in Table 2 (Appendix) and the results are shown graphically in Figure 1.

Uterine weight. The mean response to HCG was significantly greater than to PMS. The slopes of the log dose-log response lines were similar except for a significantly lower slope for the HCG at the evening injection, which occurred because in the evening the lowest level was sufficient to give a maximal response. Time to time differences in response, that is time of day and replications, were significant and there was no interaction

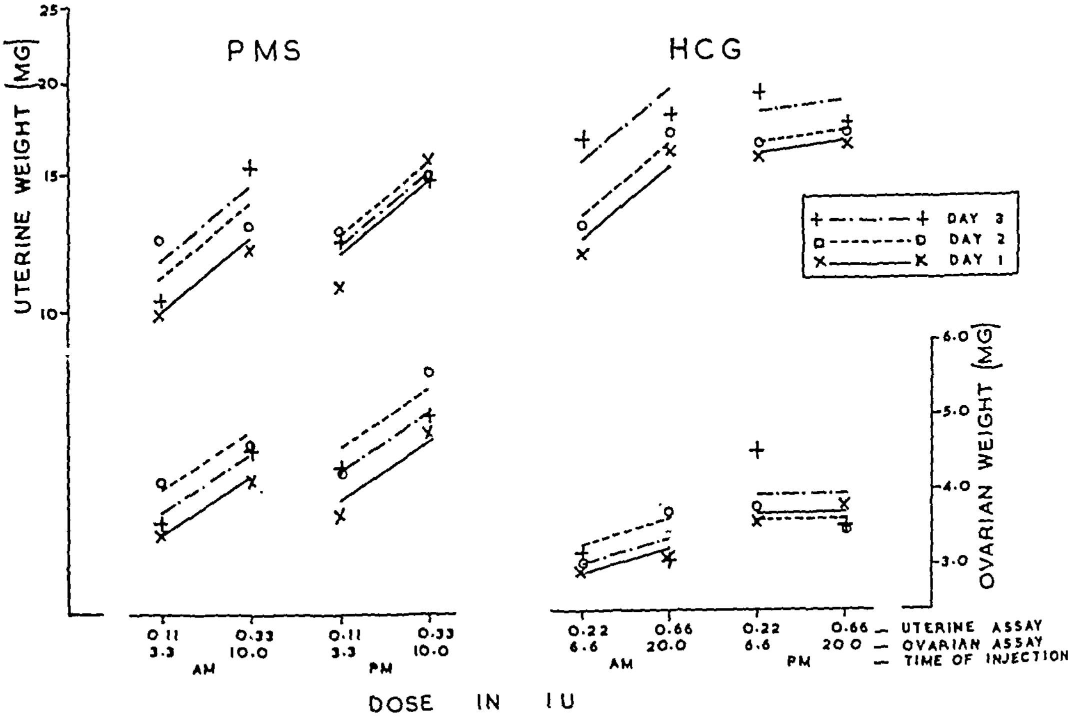

FIG. 1. The results of Experiment 1; showing diurnal variation in uterine and ovarian weights 24 hours after an injection of either PMS or HCG. Three replicates on successive days.

between the two, but it cannot be concluded from the analysis in Table 2 that differences in response between morning and evening injections were not entirely due to a linear time trend in sensitivity of the test animals. Hence it was important to examine the data further to establish the extent of time trends which were independent of time of day trends. This was done by estimating the regressions of time on response for each of the two sources of variation and comparing them in a t-test. There was a significant difference in response between morning and evening injections in spite of the underlying increase in sensitivity over the three day period ($t_{(127)}=2.24$; $P<0.05$).

The effect of storing the solutions in a refrigerator for 48 hours was a significant reduction in the response of the HCG preparations only, indicating deterioration over the period. Lamond (11) and Claringbold and Lamond (4) could not find evidence of deterioration in HCG substances when kept for much longer periods than in this experiment. However the preparations were stored at high concentrations whereas the solutions which deteriorated in the present experiment were at low concentrations.

Ovarian weight. Again it was apparent that there was no interaction between time of day and replication and as with the uterine responses it has been possible to show that there was a significant diurnal difference in response ($t_{(127)}=2.69$, $P<0.01$). No significant difference was found between the stored and fresh materials on Day 3.

Experiment II

In Experiment I the mice were killed 24 hours after the injection of gonadotropin—a time when both uteri and ovaries would be in the rapid growth phase. In view of the greater response in uteri and ovaries which resulted from the evening injection of either gonadotropin, it was thought advisable to determine if this were due merely to a faster initial rate of growth or to an actual augmentation of the final response. The uterine weight response is maximal by 40–48 hours (4) and the ovarian weight response to HCG and PMS at the dosage levels used in this experiment is maximal by 40–60 hours (unpublished data). Structural changes such as the formation of haemorrhagic follicles and corpora lutea occur in the ovaries after 60 hours. Therefore, in Experiment II the same four levels of each gonadotropin were administered to groups of randomly selected mice (five per group) which were then killed 24 or 48 hours later. The mice were weaned one hour before injection and the injections were given at 8–30 P.M. on one day and 8–30 A.M. the next day.

The data for both uterine and ovarian weights at 24 and at 48 hours were analysed separately; the experimental design thus consisted of four 4×2 factorials carried out concurrently. As in Experiment I the group totals were corrected for concomitant variation due to body weight differences and the corrected totals used in the analyses of variance which are summarized in Table 3 (Appendix). The results are shown graphically in Figure 2.

Uterine weights. At 24 hours and 48 hours the mean response to HCG was greater than to PMS. This indicated that the levels selected were relatively more potent than the corresponding levels of PMS. The slope of the

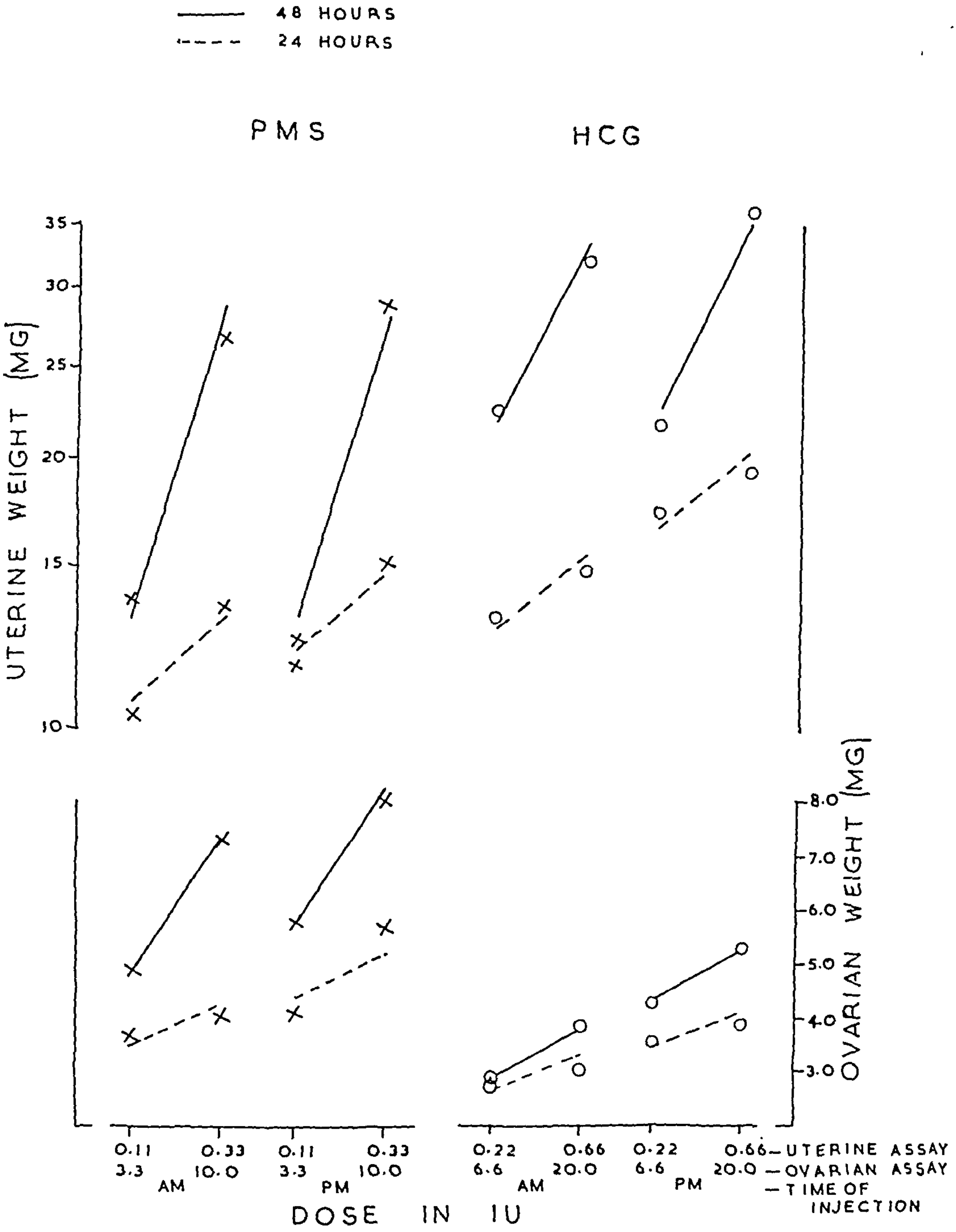

FIG. 2. The results of Experiment 2; showing diurnal variation in both uterine and ovarian weights at 24 hours, but not in uterine weights at 48 hours after an injection of PMS or HCG.

log dose-log response lines at 24 hours were similar, and as in Experiment I, the evening response was significantly greater than the morning response for both PMS and HCG. However by 48 hours when the uteri might be considered to have ceased growing there was no significant difference for either substance. Since all mice were injected over the same period of time it is clear that time changes in sensitivity, apart from the evening versus morning differences, cannot have accounted for significant differences at 24 hours followed by no differences at 48 hours.

Ovarian weights. At 24 hours the mean response to PMS was significantly greater than to HCG, and the response to the evening injection of both substances was greater than to the morning injection. No difference in the slope of the log dose-ovarian weight response lines was demonstrated. At 48 hours the mean response to PMS was much greater than to HCG and the slope of the dose-response lines was also steeper. There was a significantly greater response to the evening injection of both gonadotropins.

Experiment III

The extent of the period of altered response in various groups of mice injected at different times of the day was examined; the mice were killed 24 hours after the injection. Mice of three different ages were used in three experiments carried out in successive weeks.

(a) Mice 14–17 days old, weighing 8–11 gm. were injected immediately after weaning. The times of injection were 9 A.M., 1 P.M., 5 P.M., 9 P.M., 1 A.M. and 5 A.M. Two dosage levels of PMS, 0.5 I.U. and 5.0 I.U. were given to groups of five mice at each time.

(b) Mice 20–22 days old, weighing 10–14 gm., were injected immediately after weaning. The times of injection were the same as those in III (a). Two dosage levels of PMS, 1.0 I.U. and 10.0 I.U., were given to groups of five mice at each time.

(c) Mice 40–42 days old, weighing 13–17 gm., that had been weaned approximately three weeks prior to the experiment were used. One level of PMS, 1.0 I.U. was given to groups of 7 mice at 8–30 A.M., 12–30 A.M., 4–30 P.M., 8–30 P.M. and 12–30 P.M.

The body weights referred to were the weights recorded at slaughter. In (a) and (b) these weights were similar to weaning weights whereas in (c), in order to reduce variability in response (unpublished observations), the mice were without food for the 24 hours of the test. Since these mice lost an average of 2 gm. in this period the actual weight at commencement would have been 15–19 gm.

Both uterine and ovarian weights were recorded for each mouse. The data were analysed as in the previous experiments, the analyses of variance are summarized in Table 4 (Appendix) and the results are shown graphically in Figure 3. The results refer only to the uterine weights since the ovarian weights gave similar results.

Mice 14–17 days old did not show significant diurnal differences in response in either uterine or ovarian weights. Slightly older mice, however, exhibited a period of altered responsiveness in uteri and ovaries extending over the twelve hours from midday to midnight. In the mice which were approaching puberty the period of altered sensitivity was reduced to a few hours in the mid-afternoon. It should be mentioned that the ovaries in the youngest mice responded poorly; they were only slightly above control values even with the higher dosage. This undoubtedly accounted for the dose difference in the uterine response in these mice, whereas in mice a few days older the ovaries responded well to both levels and the uterine weight responses were maximal.

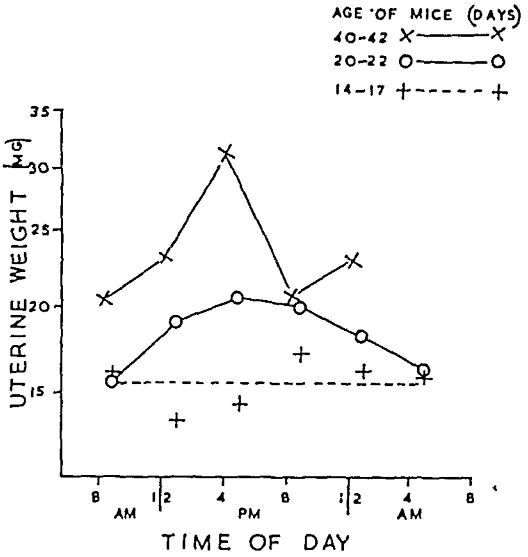

FIG. 3. The effect of age on diurnal variation in uterine weight response 24 hours after an injection of PMS.

Experiment IV

It seemed likely from the results of Experiment II that the diurnal differences in the responses observed at 24 hours might have been due to an earlier start of growth following the evening injection. An attempt was made to test this hypothesis by establishing the time-response relationships for both uterine and ovarian weights over the period of rapid growth. Two experiments were conducted:

(a) Groups of five mice were injected with 1.0 I.U. or 10.0 I.U. PMS and killed 15, 20, 25 and 30 hours later. The times of injection were 9 A.M. and 9 P.M. the day after the mice were weaned.

(b) Groups of four mice were injected with 1.0 I.U. or 10.0 I.U. HCG and killed 15, 20, 25 and 30 hours later. The times of injection were 4 P.M. the day of weaning and 6–30 A.M. the following day.

The analyses of variance are summarized in Table 5 (Appendix) and the results shown graphically in Figure 4. The mean uterine response over the period 15–25 hours for HCG and 20–30 hours for PMS was significantly greater to the evening injection than to the morning injection. The time-log uterine weight lines were linear and parallel in each case. Thus the uteri commenced rapid growth earlier after evening injection and this advantage was apparently maintained throughout the greater part of the growth period. It is of interest that after an injection of HCG the rapid growth phase appeared to commence earlier than after PMS. Since the experiments were conducted on different days it is not possible to be certain of this, however. There were no significant differences in the responses to the dosage levels for each gonadotropin, each being maximal for uterine weight, hence the data were combined for the purposes of graphical representation in Figure 4.

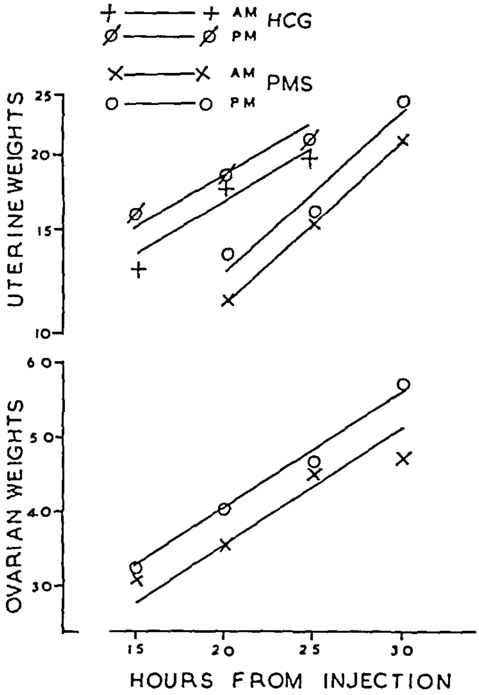

FIG. 4. Time-response relationship for uterine and ovarian weights following a single injection of HCG or PMS.

Only the data for the higher dosage level of PMS, (10.0 I.U.), have been included in the analysis of ovarian weights. The responses to 1.0 I.U. PMS and 1.0 I.U. HCG were minimal, and the ovarian responses to 10.0 I.U. HCG were not sufficiently great to show significant effects with four mice per group. The response to 10.0 I.U. PMS was apparently linear over the period examined, and as with the uterine weights, the ovarian weight response to the evening injection was significantly greater than that to the morning injection, indicating that there was less delay before the commencement of ovarian growth after the evening injection.

Experiment V

It was of some interest to determine if the diurnal variation in ovarian and uterine response was due to a diurnal rhythm of ovarian and perhaps also, uterine, responsiveness. These possibilities were examined in the following way:

(a) Forty-five mice, 18–20 days old and 10–12 gm. body weight, were hypophysectomized at 10 A.M.–12 noon, by a method described elsewhere (13). Eleven mice were each injected with 25 I.U. PMS at 9 A.M. on the second day after the operation, a further eleven at 7 P.M., and the remainder at similar times on the third day. Uterine weights were recorded 22 hours after the injection. The hypophyseal fossa of each mouse after fixation in formalin was examined using a binocular microscope. The mice in which pituitary remnants were observed were not included in the analysis.

In each of the four groups (2 times of day × 2 days) there were nine mice in which the pituitary had been completely removed. The analysis of variance (Table 6, Appendix) shows that there were no significant differences between responses at the different times of injection.

(b) Groups of 12 mice, half of which were ovariectomized, were injected with 2 μg. or 20 μg. oestradiol in water and killed 24 hours later. They were injected at 8–15 A.M. or at 4 P.M. the second day after weaning. Ovariectomy was performed one day after weaning. Body weights were 10–13 gm. at time of killing. The analysis of variance presented in Table 6 shows that there was no diurnal difference in the mean response in the ovariectomized mice or the normals. There was however, a significantly greater mean response in the ovariectomized mice. It is possible that the anesthesia caused liver damage which resulted in an enhancement of the effect of oestradiol through less efficient detoxication.

(c) Experiment V (b) was repeated without ovariectomy but the mice were killed at 16 hours. This was done in case there was an early diurnal difference which might have been eliminated at 24 hours. The mice were injected immediately after weaning. The results are not presented in detail since there was again no significant diurnal difference in the uterine response.

In both normal and ovariectomized animals the response of the uterus to injected oestrogen was thus independent of the time of injection.

Experiment VI

Since it seemed that the factor responsible for the diurnal variation on uterine and ovarian response was of pituitary origin it was thought likely that an extract of pituitary tissue might abolish the observed diurnal difference. It was also thought advisable to determine whether large doses of

PMS and HCG would affect the response. In addition an extract of postmenopausal women's urine was tested.

Five groups of each eight mice were injected at either 9 A.M. or 7 P.M. in either of two successive days. They were weaned 24 hours before injection and killed 21 hours after the injection. The injections at each time consisted of: HCG—0.3 I.U. (uterine weights) and 30.0 I.U. (uterine and ovarian weights); PMS—as for HCG; sheep anterior pituitary extract, fraction II, AP—3 mg. (uterine weights). Body weights were recorded at death.

The low level of HCG and PMS was given as a control; the results of the analysis of variance (Table 7, Appendix) show that there was a significant difference between A.M. and P.M. injections each day and, unlike Experiment I, there was no significant day-to-day difference in response. The dose of AP was approximately equivalent to each of the low levels of HCG and PMS. There were no significant differences between times of day or between days. The analysis of the data of the high levels of PMS and HCG is also shown in this table; there were no significant time-to-time differences for either uterine or ovarian weight. There was however a significantly greater mean uterine and ovarian response with PMS.

In another experiment the above results were confirmed, using an 0.5 *M* buffered saline extract of sheep pituitary glands. The preparation of postmenopausal women's urine (HMG) gave similar results to AP.

DISCUSSION

Mice injected with moderate doses of PMS and HCG in the evening clearly give greater uterine and ovarian responses 24 hours later than do mice treated similarly but injected in the morning. The phenomenon is exhibited equally well with both PMS and HCG. In addition, the evening injection causes an earlier beginning of ovarian and uterine growth and also results in significantly greater ovarian, but not uterine, weights at 48 hours.

An important problem in biological studies is the variability which exists between test subjects. The introduction of appropriate statistical measures will often bring such variation under the control of the operator. Nevertheless such measures may add greatly to the complexity of experiments which are conducted over a period because time-to-time variation is normally unpredictable and occasionally large. It is therefore of considerable importance that time-to-time variation in response to gonadotropin has been shown to have a diurnal component.

There are several mechanisms that might be responsible for the diurnal variation in response to PMS and HCG. The results of Experiments V (b) and (c) seem to eliminate the possibility that there is a diurnal variation in uterine responsiveness, and the findings in hypophysectomized animals (Experiment V (a)) and the absence of a diurnal difference with pituitary extracts suggest that the phenomenon is not a manifestation of an intrinsic daily rhythm of sensitivity in the ovary, but that the fluctuations in ovarian and uterine responses find their origin in the pituitary gland. The results obtained with hypophysectomized animals are by no means clear cut however, for the sensitivity of the ovary to PMS in such animals is much lower than in intact animals (13). Nevertheless it is to be expected from a number of other studies (2, 6, 7, 8, 10) that such a diurnal rhythm would have a neuro-humoral mechanism involving the hypothalamus and the pituitary gland.

The ovaries and uteri of immature mice begin to exhibit increases in weight between 15 and 20 hours after the injection of gonadotropin (5) (and present data, Experiment IV). However the delay between injection of gonadotropin and the beginning of uterine and ovarian growth has been found to show significant variation with time of day, being longer when injection is given in the morning. Once it has begun, the rate of increase in weight of both organs does not depend on time of day. The finding that the increased delay after the morning injection is not abolished until the dosage levels of HCG and PMS are 20 or more times greater than that needed to produce a maximal uterine weight response, is of considerable significance.

It seems to indicate that there is a diurnal fluctuation in the output of a pituitary factor that is qualitatively different from the gonadotropin(s) assayed by the ovarian or uterine weight tests (FSH-like and LH-like gonadotropins). A diurnal variation in response of the uterus was not observed when doses of sheep anterior pituitary and human post-menopausal gonadotrophin which were approximately equivalent to 1 I.U. HCG or PMS as judged by the uterine response, were used, hence the diurnally fluctuating factor presumably cannot be the gonadotropin (14) measured by the uterine weight response. Since HCG and PMS appear to have brought about similar response trends to low and high levels and since PMS is rich in a gonadotropin biologically similar to FSH, it seems that the diurnally fluctuating factor cannot be FSH-like either. The results may therefore indicate the presence of a pituitary factor different from FSH and LH, which is necessary for steroid production or ovarian growth. In the evening the pituitary gland presumably produces more of this factor. Whether it is a gonadotropin however is open to question. At least it does not appear to be necessary for uterine response to oestrogen for not only was there no diurnal variation in response to oestradiol (Experiment V b and c) but hypophysectomy does not decrease the sensitivity of the uterus to oestradiol (Lamond, unpublished observations).

Another explanation of the present findings is suggested by the recent results of Lamond and Emmens (13) who showed that responses to the placental gonadotropins HCG and PMS are apparently limited by endogenous gonadotropin production. If the animal's own pituitary gland responds to the injection of HCG or PMS then it may be that there is a diurnal variation in the rapidity of this response. A direct action of the placental gonadotropins on the neuro-hypophyseal axis has not, however, been demonstrated but may well repay further investigation. Alternatively the endogenous gonadotropins may not act on the ovary in a manner different from PMS and HCG but the amount produced may be considerably in excess of the effective amount of exogenous gonadotropin.

The time of day when the gonadotropin was injected had no influence on the uterine weight 48 hours later (i.e. at a time when uterine growth had ceased) but there was a difference at 48 hours in ovarian weights. The reason for the divergence between these two criteria of ovarian response is not apparent.

The results of Experiment III call for special mention. It is apparent that mice 14–17 days old are less sensitive to gonadotropin than mice 3–4 days older. Similar findings have been reported by Ortiz (15) for the rat and hamster. In the mice the lower sensitivity was particularly evident in the ovarian weight responses, which were not significantly greater than the controls. In the slightly older and bigger mice, not only was there a response in both uteri and ovaries, but there was also a period of increased ovarian responsiveness over the 12 hours from midday to midnight. In mice two weeks older and an estimated two weeks before puberty, this period was considerably shorter, being confined approximately to 2 P.M.–6 P.M. Hence in the strain of mice used in these experiments there seems to have been a gradual development of an overall responsiveness to HCG and PMS, and this has a diurnal component which becomes more closely confined to the mid-afternoon as the mice approach puberty. Pfeiffer (16) postulated that the sex type of the pituitary gland is not genetically determined but that its differentiation is dependent on the type of gonad present. It is possible that this differentiation is simply the development of a diurnal rhythm.

Acknowledgment

We wish to thank Professor C. W. Emmens and Dr. P. J. Claringbold for assistance and advice. We are indebted to Dr. K. A. Ferguson, C.S.I.R.O., Prospect, N.S.W., for the pituitary gland extracts and to Mr. L. Martin of this Department for the HMG. One of us (D.R.L.) is indebted to the C.S.I.R.O. for a Senior Post-graduate Studentship. The work was aided by grants from the Commonwealth Bank of Australia and the Wool Industry Fund.

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APPENDIX

Table 2. Analyses of variance of the data obtained in experiment 1

Mean squares headed (i) are comparisons between fresh preparations only and (ii) are comparisons between stored and fresh preparations injected on Day 3. The non-significant interactions in this and following experiments are pooled to form a residual term, which is not used in the estimate of error.

Source of variation	D.F.	Mean square			
		Uterine weight		Ovarian weight	
		(i)	(ii)	(i)	(ii)
Preparations (P)	1	3203***	1629***	22.88***	15.58***
Doses (D)	1	1613***	1272***	4.88***	3.66***
Time of day (T)	1	780**	557**	9.86***	16.47***
Replications (R)					
Linear	1	714**		1.65*	
Quadratic	1	6		1.62*	
Stored v Fresh (S)	1		447*		0.06
Interactions					
P×D	1	224	525**	7.40**	3.92**
P×D×T	1	326*		2.00*	
P×S	1		447*		0.11
Remainder	16	70		0.44	
	9		52		0.37
Error	127	71.3		0.37	

*** $P<0.001$.
** $0.001<P<0.01$.
* $0.01<P<0.05$.

Table 3. Analyses of variance of the data obtained in experiment II

Source of variation	D.F.	Mean square			
		Uterine weight		Ovarian weight	
		At 24 hr.	At 48 hr.	At 24 hr.	At 48 hr.
Preparations (P)	1	1134***	2592***	11.77***	55.56***
Doses (D)	1	640**	6864***	3.91**	28.20***
Time of day	1	706**	14	8.01***	19.84***
Interactions					
P×D	1		384*		5.48**
Remainder	4	55		0.78	
	3		53		0.32
Error	31	78.9	88.7	0.44	0.69

Table 4. Analyses of variance of the data obtained in experiment III

Source of variation	D.F.	Mean square		
		(a)	(b)	(c)
Time of day				
Linear	1	183	0	96
Quadratic	1	10	876***	433
Cubic	1	56	26	245
Quartic	1	17	1	1026**
Quintic	1	38	0	
Doses	1	2208***	70	
Interaction	5	59	30	
Error	47	71.6	42.3	
	29			133.7

Table 5. Analyses of variance of the data obtained in experiment IV

Source of variation	D.F.	Mean square		
		Uterine weights		Ovarian weights
		(a)	(b)	(a)
Time of day	1	465**	305**	2.40*
Time of killing				
Linear	1	7156***	1740***	24.22***
Quadratic	1	147	4	0.11
Cubic	1			0.10
Doses	1	184	99	
Interaction	7	31	63	
	3			0.33
Error	47	47		
	35		38	
	31			0.32

Table 6. Analyses of variance of the results of experiments V (a) and V (b)

	Source of Variation	D.F.	Mean square
V (a)	Day 1 v Day 2	1	54
	A.M. v P.M.	1	22
	Interaction	1	4
	Error	31	37.6
V (b)	Normal v ovariectomized	1	999***
	A.M. v P.M.	1	1
	Between doses	1	2338***
	Interaction	4	40
	Error	39	43.1

Table 7. Analyses of variance of the data obtained in experiment VI

Source of variation	D.F.	Mean square			
		Uterine weights			Ovarian weights
		Low levels HCG, PMS	High levels HCG, PMS	AP	High levels HCG, PMS
Preparations	1	56	1156***		10.32***
Day 1 v day 2	1	225	0	145	0.88
A.M. v P.M.	1	1541***	56	45	0.70
Interaction	4	9	94		0.73
	1			18	
Error	55	89.5	63.8		0.35
	27			63.2	

A COMPARISON OF THE EFFECTS OF *IN VIVO* AND *IN VITRO* THYROID HORMONES UPON THE OXYGEN CONSUMPTION OF RAT KIDNEY SLICES[1]

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ABSTRACT

Immediately after removal of one kidney from thyroidectomized rats a large amount of thyroxine or triiodothyronine was injected intravenously. Slices made from the remaining kidney, removed 10 minutes later, had QO_2 levels significantly higher than control values after storage at 4° C for 48 hours in alanine buffer. After the injection of labeled thyroxine the I^{131} content of the injected kidney corresponded to .03 μg. thyroxine per mg. wet weight. Diiodotyrosine, injected under similar conditions, did not affect the QO_2. Thyroxine added to slices from both control and injected kidneys was also effective in preserving QO_2.

MATERIALS with thyroid hormone activity are effective *in vitro* if added to ruptured cells (1), to their separated components (2, 3), or to ascites tumor tissue (4). Thibault (5) and Barker (6) have studied the effect of added thyronine derivatives upon the oxygen consumption of slices made from the kidneys of thyroidectomized rats. The slices were stored at 4° C between daily measurements of oxygen consumption (QO_2) at 37° C, and under these conditions the metabolically active thyronine derivatives delayed the expected decline in QO_2 with time to a striking degree.

The present experiments were designed to determine whether a similar delayed decline in QO_2 with time is present if the thyroid hormones reach the kidney via the circulatory system and the amount of thyroid hormone that is effective under these *in vivo* conditions.

METHODS

Male albino rats from the Charles River Breeding Laboratories were surgically thyroidectomized when body weight was 50 to 100 gm., at least two months before the experiment. They received Purina laboratory chow and tap water *ad libitum*. At the time of the experiment the animals were anaesthetized with ether, and through a mid-abdominal incision the right renal artery and vein were clamped with a hemostat. The right kidney was then removed and cooled on ice. 1 mg. of the material being tested, dissolved in dilute NaOH, was injected into the inferior vena cava in a volume of 0.5 cc.,

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and 10 minutes later the other kidney was removed. In a moist chamber two slices were made from the lateral aspects of each kidney with a Stadie-Riggs microtome (four slices per kidney), and in order to diminish error due to variable amounts of cortex and medulla, half of an inner and of an outer slice were weighed together and placed in each of two Warburg vessels. The buffer used throughout contained .12*M* NaCl, .003*M* KCl, .0012*M* $MgSO_4$, .017*M* DL-alanine (pH 7.8), and .01*M* glucose, final pH 7.2. The gas phase was oxygen, temperature 37° C, shaking speed 120 strokes per minute. QO_2 is based upon the first 30 minutes of observation, although the vessels were kept at 37° C for approximately 70 minutes daily. Between oxygen consumption measurements they were covered with paraffin film and stored at 4° C. When labeled thyroxine was used, radioactivity was measured by placing slices in tubes of cold buffer in a well-type scintillation counter prior to transferring them to Warburg vessels. DL-thyroxine was obtained from Nutritional Biochemicals Corp., 3,5,3′ L-triiodothyronine from Dr. A. E. Heming of the Smith, Kline, and French Corp., 3,5, diiodo-L-tyrosine from Eastman Kodak Co., and L-thyroxine labeled with I^{131} from Abbott Laboratories, Oak Ridge.

RESULTS

The table summarizes, with statistical evaluation, the QO_2 results measured at successive 24 hour intervals following the removal of the tissue. It can be seen that highly significant changes are produced by injection of materials with known metabolic activity. In the case of DL-thyroxine, the changes are seen after 48 hours, but they appear in 24 hours when 3,5,3′ L-triiodothyronine is used, and are absent in tissues taken from animals previously injected with diiodo-L-tyrosine. The same data are included in the figures as black bars. Slices from each kidney were also placed in contact with L-thyroxine, 10 μg. per cc. of buffer. In the figures the QO_2 values so obtained are shown by white bars. It can be seen in Figure 1 that after 48 hours in the presence of added thyroxine the QO_2 of the tissue remains high, and that this result is not affected by the prior injection of diiodo-L-tyrosine. Figures 2 and 3 show that a further increase can be produced by the *in vitro* exposure to thyroxine, even though the animal has previously been injected with a metabolically active hormone preparation. The increases in QO_2 from *in vitro* contact with thyroid hormone after 48 and 72 hours are all significant with a P value of less than .001.

Ten minutes after the injection of DL-thyroxine to which a small amount of I^{131} labelled L-thyroxine had been added, the tissue I^{131} level corresponded to .03 ± .004 (S.E.) μg. thyroxine per mg. wet weight (4 measurements on each of 9 animals).

DISCUSSION

The method of Barker (6) represents one of the few demonstrations of an *in vitro* effect of materials with thyroid hormone activity upon excised tissue containing whole cells. The complex changes which occur in tissue permeability following excision (7) might be thought to favor the entrance of hormone into the cell, and indeed, in a recent note (8) Barker and Etling have shown that added thyroxine is rapidly taken up by excised kid-

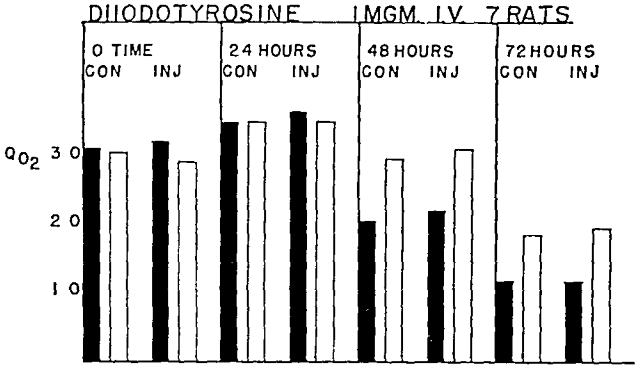

FIG. 1. Results of daily measurement of QO_2 following injection of diiodotyrosine. The black bars show the mean QO_2 for slices from control and injected kidneys suspended in buffer alone, the white bars show the mean QO_2 for slices from the same kidneys suspended in buffer containing 10 micrograms L-thyroxine per ml.

QO_2 OF SLICES MADE FROM CONTROL AND INJECTED KIDNEYS
1 MG. OF EACH COMPOUND INJECTED INTRAVENOUSLY

Time in hours	DL-thyroxine (16 rats)			L-triiodothyronine (10)			diiodotyrosine (7)		
	Con.	Inject.	P	Con	Inject	P	Con	Inject	P
0	3 04 ± 10*	2 90 ± 09	> 1	3 26 ± 11	3 20 ± 10	> 1	3 16 ± 08	3 25 ± 09	> 1
24	3 47 ± 10	3 41 ± 09	> 1	3 36 ± 07	3 60 ± 09	< 05	3 52 ± 12	3 68 ± 08	> 1
48	1 96 ± 07	2 35 ± 05	< 001	1 97 ± 03	2 32 ± 06	< 001	2 09 ± 11	2 24 ± 08	> 1
72	1 08 ± 04	1 08 ± 05	—	1 04 ± 07	1 22 ± 06	> 05	1 21 ± 09	1 20 ± 08	> 1

* Standard Error.

ney tissue. The present experiments, in which the hormones reach the tissue via the vascular system, demonstrate that the swelling of cells following excision need not precede contact with thyroid hormones for a demonstrable effect in preserving oxygen consumption, but add no information on the question of later tissue swelling as a consequence of contact with these compounds.

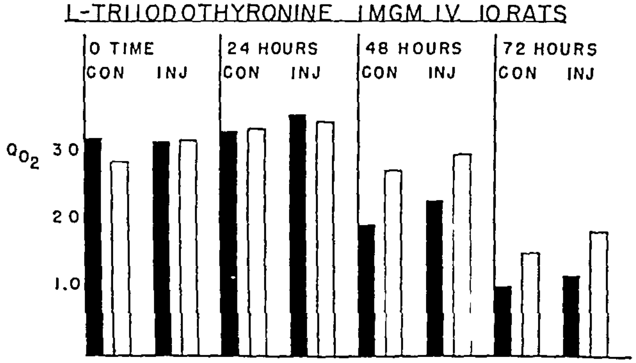

FIG. 2. Results of daily measurement of QO_2 following injection of 3,5,3′ L-triiodothyronine.

Legend as in Figure 1.

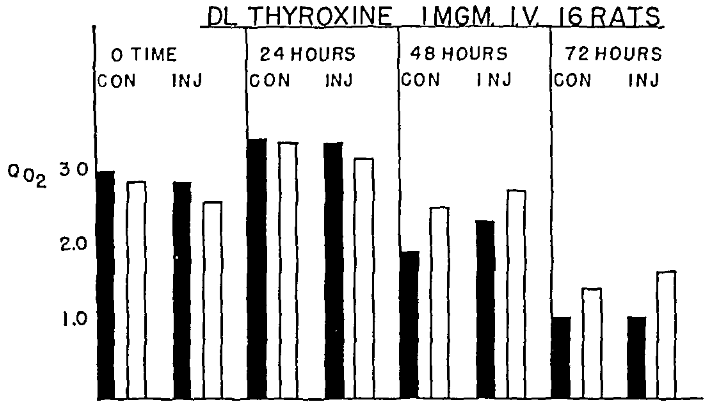

FIG. 3. Results of daily measurement of QO_2 following injection of DL-thyroxine. Legend as in Figure 1.

Since the effects of thyroid hormones reaching the cells via the vascular system and *in vitro* are additive, it seems probable that .03 μg. of thyroxine per mg. of tissue is insufficient under the present conditions to produce a maximum tissue response. This impression has been confirmed in a few experiments by noting a larger response after the injection of larger amounts of hormone.

Each flask in the present studies contained an average of 75 mg. of tissue in 3 cc. of buffer. Assuming that thyroxine added *in vitro* is all rapidly bound by kidney tissue (8), the experiments with injected hormone correspond to about 2 μg. of added thyroxine per vessel. This is close to the minimum amount reported to be active *in vitro* (9). Although experimental conditions are strikingly different, it is of interest to note that this concentration of thyroxine, $10^{-6}M$, has been shown to uncouple oxidation from phosphorylation in isolated mitochondria *in vitro* (10) but is 100 times the concentration required for the *in vitro* production of mitochondrial swelling (11).

In every experiment it can be seen that the QO_2 of kidney slices increases during the first 24 hours after excision. This phenomenon, which has also been observed by Barker (9), appears to be somewhat more striking in the presence of added or injected thyroid hormones than in their absence. A similar increase is seen following the injection of diiodotyrosine, however, which makes the relationship between this finding and thyroid hormone action questionable. The initial lowered QO_2 may be the result of impaired diffusion from compression of tissue during slicing, though we have not performed experiments to check this point.

In the present experiments there is a more rapid effect observed after the injection of triiodothyronine than after a similar amount of DL-thyroxine. On the basis of the rapid onset of metabolic activity following the injection of triiodothyronine (12), and the isolation of this compound from thyroid tissue after injection of I^{131} (13) it has been suggested that triiodothyronine is a step closer to the form of the thyroid hormone which acts upon the cell. It should be pointed out, however, that the present experiments could well be explained on the basis of the rapid penetration and high tissue hormone concentration found after the injection of triiodothyronine as contrasted to thyroxine (14). The many possible variations in the present technique remain to be explored.

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INVESTIGATION OF THE RELEASE OF ACTH[1]

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ABSTRACT

The ACTH-releasing activity of portal vessel plasma, as estimated by adrenal ascorbic acid depletion in the steroid-depressed rat, was present in protein fraction III_0. The reduction in adrenal ascorbic acid concentration following injection of III_0 was not attributable to the presence of ACTH in this protein fraction since III_0 was inactive in the hypophysectomized rat. The remaining protein fractions had no significant effect on adrenal ascorbic acid concentration. The activity attributable to III_0 was lost during dialysis when the protein fraction was dissolved in a buffer solution containing 15.2% ethanol. This suggested either destruction of the active component or dissociation of a dialyzable component from the carrier protein.

Fraction III_0 from carotid artery plasma was inactive regardless of whether the blood was collected rapidly, slowly, or allowed to remain mixed with macerated skeletal muscle for two hours at 0° C.

The ACTH-releasing activity of portal plasma III_0 was not attributable to contamination with nor-epinephrine, epinephrine, histamine, or serotonin. Similarly, injection of NaCl solutions of different concentrations were ineffective.

THE primary cause of variation in the rate of ACTH discharge from the anterior pituitary in response to an endless array of environmental changes remains an enigma, and it is to be emphasized that the circumstances which decrease the rate of ACTH discharge are no less important in homeostasis than are those circumstances which increase ACTH release. Admittedly, it is difficult to ascribe specificity to a particular stimulus for the latter; however, determination of the effective site of action of an inhibitor of ACTH release is no less difficult. The results of some investigations suggest the involvement of a neurohumoral agent, "corticotrophin-releasing factor—CRF" (1), but a discordant view regarding the uniqueness of such a substance is not without significance. Such divergent views exist not only because of possible lack of quantity and purity of CRF but also because of lack of proven specificity and defined limits of applicability for the assay system used in estimating CRF activity. The difficulties inherent in the assay of CRF have been considered in a recent review (2). Nonethe-

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less, previous reports which suggested that blood collecting in the sella area following hypophysectomy (3, 4) and blood draining the brain (5, 6) accelerated the release of ACTH prompted further consideration of this problem. Preliminary reports of part of this work have been previously presented (7, 8).

MATERIALS AND METHODS

Plasma used in this investigation was obtained from blood of mongrel dogs of both sexes. Blood for experimental purposes was collected by aspirating that which accumulated in the sella area following removal of the pituitary, the source of this blood being in part the hypophyseal portal vessels. The procedure employed here was the same as that previously described (3). The plasma was stored at −20° C until used. The procedure followed in fractionation of the plasma proteins and the terminology used in referring to the different fractions was identical to that used by Cohn *et al.* (9). Dialysis was carried out with stirring at 4° C for approximately 2 hours utilizing Visking dialysis tubing,[2] size 24/32 with a specified average pore radius of 24 Å. In all cases the sample was dried by lyophilization before use in assay, and the dried sample was dissolved in 4 cc. of distilled water for injection.

Male albino rats were used in the assays. The hypophysectomized rat was prepared by the parapharyngeal approach and was used in the adrenal ascorbic acid depletion test 20 to 24 hours after hypophysectomy (10). The hydrocortisone-inhibited intact rat was prepared by injecting intraperitoneally 6 mg. of hydrocortisone acetate in saline per 100 gm. body weight 4 hours before use. Such an animal does not release ACTH in response to unilateral adrenalectomy (3). All solutions were administered through the tail vein in two injections of 2 cc. each. The first injection was given immediately after removal of the left adrenal, and the second was given 30 minutes later. The right adrenal was removed 90 minutes after the first injection. The first adrenal was removed under ether anesthesia; the second adrenal was removed under nembutal anesthesia. In each instance the total amount of lyophilized plasma or plasma fraction injected was equivalent to 10 cc. of original plasma.

RESULTS

The intravenous administration of lyophilized portal vessel plasma to hydrocortisone-inhibited intact rats resulted in significant reduction in adrenal ascorbic acid concentration ($P < 0.01$). Following partitioning of the portal plasma proteins into fractions IV+V+VI, I+III_3, II, $III_{1,2}$ and III_0, only fraction III_0 caused a significant reduction in adrenal ascorbic acid concentration ($P < 0.01$) (Table 1).

It has been reported (4) and confirmed in this investigation that the ACTH-releasing activity of portal vessel plasma was not affected by dialysis against 0.9% NaCl solution. However, extension of this observation to fraction III_0 must be restricted. The solution used to dissolve III_0 contained 0.6 moles glycine and 2.62 moles ethanol and was buffered with glycinate and phosphate at pH 6.8–6.9 (9). When this solution containing III_0 was lyophilized to remove the ethanol prior to dialysis against 0.9% NaCl solution, the change in adrenal ascorbic acid following injection of

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[2] Visking Corporation, Chicago, Illinois.

TABLE 1. EFFECT OF PORTAL PLASMA AND PLASMA FRACTIONS ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-TREATED RATS

Number of rats	Weight of rats (gm.)	Ascorbic acid concentration (left adrenal at 0 min.) (mg./100 gm.) mean	Final treatment (two injections i.v.—2 cc. per injection)	Ascorbic acid concentration (right adrenal at 90 min.) (mg./100 gm.) mean	Change in adrenal ascorbic acid concentration (mg./100 gm.)
24	323±11.6*	345	*0 min.*—lyophilized portal plasma equivalent to 5 cc. *30 min.*—lyophilized portal plasma equivalent to 5 cc.	261	−84±6.3
12	294±21.6	340	*0 min.*—fraction IV, V, VI equivalent to 5 cc. portal plasma *30 min.*—fraction IV, V, VI equivalent to 5 cc. portal plasma	334	− 6±6.7
12	291±12.4	333	*0 min.*—fraction I+III$_3$ equivalent to 5 cc. portal plasma *30 min.*—fraction I+III$_3$ equivalent to 5 cc. portal plasma	341	+ 8±5.9
15	295±14.6	349	*0 min.*—fraction II equivalent to 5 cc. portal plasma *30 min.*—fraction II equivalent to 5 cc. portal plasma	360	+11±4.4
2	232±13.0	370	*0 min.*—fraction III$_{1,2}$ equivalent to 5 cc. portal plasma *30 min.*—fraction III$_{1,2}$ equivalent to 5 cc. portal plasma	370	0±3.5
59	300± 7.9	342	*0 min.*—fraction III$_0$ equivalent to to 5 cc. portal plasma *30 min.*—fraction III$_0$ equivalent to 5 cc. portal plasma	316	−26±5.4**

* Mean and standard error.

** This value includes the results from all assays regardless of treatment of III$_0$. An explanation of the discrepancy between this value and that for lyophilized plasma is suggested by the data persented in Table 2.

III$_0$ was −55±9.7 mg. per 100 gm. adrenal weight for 18 determinations. However, when this same solution containing III$_0$ was dialyzed against 0.9% NaCl solution without prior lyophilization, the change in adrenal ascorbic acid following injection of III$_0$ was +1±5.1 mg. per 100 gm. adrenal weight for 18 determinations. The difference in these two means was statistically significant ($P<0.01$) (Table 2).

When III$_0$ from portal plasma was assayed in the 24-hour hypophysectomized rat and the hydrocortisone-treated rat, the changes in adrenal ascorbic acid concentration were +6±6.2 mg. per 100 gm. adrenal weight for 7 determinations and −55 mg. ±24.1 per 100 gm. adrenal weight for 6 determinations respectively. The difference in these two means was significant ($P=0.03$) (Table 2). Fraction III$_0$ used here was from a common pool which was dried by lyophilization prior to dialysis.

Plasma protein fraction III$_0$ was prepared from (a) carotid artery blood which was permitted to flow rapidly during collection, (b) carotid artery blood which was allowed to drip into an iced beaker during a 3 hour collection period, and (c) a mixture of 200 cc. of carotid artery blood and 10 gm. of macerated skeletal muscle. This mixture remained in an ice bath for one hour before separation of the plasma. The mean change in adrenal ascorbic acid for 7 determinations in each group was −3 ±4.2, +3 ±3.1, and +10 ±2.7 mg. per 100 gm. adrenal weight respectively.

As it was difficult to maintain constant osmolarity of the solutions injected during these experiments, NaCl solutions of different concentrations were given intravenously to the steroid-inhibited rat in an attempt to assess the effect of solutions of varying osmolar concentrations on the release of ACTH. After injection of 4 cc. in two 2 cc. injections of 0.45, 0.90, 2.25, 3.15 and 4.05 per cent NaCl solutions, the change in adrenal ascorbic acid was +9 ±2.0, +12 ±0.7, +1 ±3.1, +13 ±3.0 and +7 ±6.1 mg. per 100 gm. adrenal weight respectively. These observations suggested that variation in osmolarity of injected solutions within these limits had no effect on the release of ACTH in these steroid-treated rats. Following intravenous administration of 8 μg. of nor-epinephrine, 8 μg. of epinephrine and 6 mg. of histamine hydrochloride, the change in adrenal ascorbic acid concentration was +22 ±8.0, −1 ±2.2 and +7 ±5.6 mg. per 100 gm. adrenal weight respectively. The change in adrenal ascorbic acid in the steroid-treated rat injected with a total of 40 μg. of serotonin[3] was −1 ±3.3 mg. per 100 gm. adrenal weight, of 80 μg. of serotonin was 0 ±1.0 mg., of 160 μg. of serotonin was +2 ±1.6, and of 320 μg. of serotonin was −2 ±10.0.

TABLE 2. EFFECT OF DIALYSIS OF PLASMA FRACTION III_0 ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-TREATED RATS

Number of rats	Weight of rats (gm.)	Ascorbic acid concentration (left adrenal at 0 min.) (mg./100 gm.) mean	Final treatment (two injections i.v.—2 cc. per injection)	Ascorbic acid concentration (right adrenal at 90 min.) (mg./100 gm.) mean	Change in adrenal ascorbic acid concentration (mg./100 gm.)
18	316±12.6*	340	*0 min.*—fraction III_0 equivalent to 5 cc. portal plasma (dialysis without prior lyophilization) *30 min.*—fraction III_0 equivalent to 5 cc. portal plasma (dialysis without prior lyophilization)	341	+ 1±5.1
18	290±12.7	339	*0 min.*—fraction III_0 equivalent to 5 cc portal plasma (dialysis with prior lyophilization) *30 min.*—fraction III_0 equivalent to 5 cc portal plasma (dialysis with prior lyophilization)	284	−55±9.7

* Mean and standard error.

DISCUSSION

These results supported the suggestion (3, 4) that blood that drained into the sella area of the hypophysectomized dog stimulated the release of ACTH in the hydrocortisone-treated rat as estimated by adrenal ascorbic acid depletion. Following fractionation of the portal plasma proteins,

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[3] Injected as serotonin·creatinine·H_2SO_4 H_2O complex. The authors express appreciation for the generous gift of serotonin from Vismara Terapeutici. This was obtained through the California Foundation for Biochemical Research, Los Angeles.

ACTH-releasing activity was present only in fraction III_0. No change in adrenal ascorbic acid was observed following injection of an equal amount of III_0 in the hypophysectomized rat, suggesting that the source of ACTH released here as a result of injection of III_0 was the pituitary. As the likelihood existed that the ACTH-releasing activity in portal plasma was attributable to the prolonged standing of the portal vessel blood in an ice bath during the collection period, carotid artery blood was allowed to drip into a cold container during a two-hour collection period in an attempt to control this variable. Also, to account for possible tissue contamination macerated skeletal muscle, was mixed with carotid artery blood and the mixture allowed to stand in an ice bath for one hour. The injection of plasma fraction III_0 from each of these samples of carotid artery blood did not cause reduction in adrenal ascorbic acid of the steroid-depressed rat.

The loss in ACTH-releasing activity of portal plasma III_0 following dialysis when this fraction was still dissolved in a solution containing 15.2% (v/v) ethanol at pH 6.8–6.9 suggested destruction of the active component or the dissociation of a dialyzable substance from the carrier protein. Lyophilization prior to dialysis prevented such loss of activity. Since the net result of lyophilization here was the removal of ethanol and water, the latter of necessity being partly replaced to make dialysis possible, it would seem that the presence of ethanol under these conditions promoted destruction or dissociation of the active substance.

It is interesting to speculate regarding the possible identity of the active component in III_0 and DΔ, a preparation having no pressor activity (11, 12). McCann, using rats with hypothalamic lesions, was unable to separate pressor activity and ACTH-releasing activity of Pitressin (13). However, interpretation of observations regarding the role of the antidiuretic hormone as well as other substances in the regulation of ACTH release from the pituitary is complicated by the observations that Pitressin potentiated the effect of ACTH on cortical steroid production in the dog (14) and that Pitressin and arginine vasopressin caused adrenal ascorbic acid depletion in the 'decapitated' rat (15). Fraction III_0 from portal plasma is without effect on adrenal ascorbic acid in the hypophysectomized rat under the conditions of the present experiments. This does not exclude the possibility that greater quantities would be effective. Since nor-epinephrine, epinephrine, histamine, serotonin and sodium chloride solutions of varying concentrations did not significantly decrease the concentration of adrenal ascorbic acid in the hydrocortisone-inhibited intact rat, it was concluded that the ACTH-releasing activity of III_0 was not due to contamination with these substances or to different osmolarities of the injected solutions. It will be of interest to see if the results of future investigations justify the restriction of corticotrophin-releasing activity to a single substance or show it to be a property of several substances. The results of this investigation, however, are in accord with the view that a neurohumoral agent is transported to the anterior pituitary through the portal vessels and that this substance increases the rate of release of ACTH from the pituitary.

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FURTHER STUDY OF AN ACTH-RELEASING PROTEIN FROM HYPOPHYSEAL PORTAL VESSEL PLASMA[1]

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ABSTRACT

Fraction III_0 obtained from hypophyseal portal vessel plasma by the Cohn fractionation procedure possessed all of the ACTH-releasing activity of portal plasma. This fraction consisted of three major protein components with isoelectric points at pH 4.4, pH 5.1 and pH 6.6. Separation of the components of this fraction by starch column electrophoresis yielded an active peak with a mobility in veronal buffer (pH 8.6; $\mu = 0.1$) of $-1.91 \pm .41$ cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^{-5}$.

The activity associated with the proteins of fraction III_0 was destroyed by incubation with trypsin and pepsin and by refluxing with 0.1 N NaOH. It was not destroyed by two hours of refluxing with 0.1 N HCl.

Preliminary results indicated that the active substance could be dialyzed away from the proteins in distilled water if the lyophilized portal plasma was dissolved in a Cohn Buffer at pH 6.9. This would suggest that this plasma protein serves in a transport role for an ACTH-releasing substance in portal vessel blood.

THE ACTH-releasing activity of hypophyseal portal vessel plasma (1, 2) was found in one protein fraction (3). This investigation deals with further purification of this protein fraction, electrophoretic characterization of its components, and the determination of some of its properties which may help to elucidate its role in the release of ACTH. Preliminary results of part of this work have been previously reported (4, 5).

MATERIALS AND METHODS

Plasma used in this investigation was obtained from blood of mongrel dogs of both sexes. Blood for experimental purposes was collected by aspirating that which accumulated in the sella area following removal of the pituitary, the source of this blood being in part the hypophyseal portal vessels. The procedure employed here was the same as that previously described (1). The plasma was stored at −20° C until used. It was fractionated by the low temperature alcohol fractionation method of Cohn *et al.* (6), and the terminology used in identifying the different fractions was the same as that used by Cohn. The use of the hydrocortisone-inhibited intact rat in estimating ACTH-

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releasing activity was the same as that previously described (1), and depletion of adrenal ascorbic acid was used as the criterion of ACTH release (7).

Electrophoretic mobility of the III_0 fractions was determined by moving boundary electrophoresis of 1% protein solutions. Protein concentration was measured by a biuret method (8). The solutions were dialyzed against the following buffers of 0.1 ionic strength (μ): acetate at pH 3.6 and pH 4.5, cacodylate at pH 5.6, phosphate at pH 6.8 and veronal at pH 8.6; and electrophoresis was carried out in a Perkin-Elmer model 38A electrophoresis apparatus. Buffer resistance was measured at 0° C with a standard conductivity cell and an impedance bridge.

The III_0 proteins were further separated by starch zone electrophoresis (9) using acetate buffer at pH 3.6 ($\mu = 0.1$), 150–160 volts and 15–20 milliamperes for periods ranging from 22 to 48 hours. Analysis for protein in aliquots from 1 cm. segments of the column was by the Folin-Lowry procedure (10). Segments corresponding to a peak were pooled, dialyzed, lyophilized and assayed for ACTH-releasing activity. The mobility of pooled active peaks from the starch columns was determined by moving boundary electrophoresis in veronal buffer at pH 8.6 ($\mu = 0.1$).

All dialyses in this investigation were carried out with stirring at 4° C utilizing Visking dialysis tubing[2], size 24/32 with a specified average pore radius of 24 Å.

For tryptic and peptic digestions of III_0, aliquots equivalent to 10 cc. of original plasma and containing 51 mg. of protein were dialyzed for two hours against two changes of 1.5 liters of 0.9% NaCl solution and then lyophilized. One aliquot was then assayed again for activity. Another aliquot was dissolved in 50 cc. of 0.5% Na_2CO_3 solution, adjusted to pH 8.3 with HCl and mixed with 5 mg. of Difco trypsin. A third aliquot was dissolved in 50 cc. of 0.1 N HCl and mixed with 5 mg. of Difco pepsin. Incubation was carried out with shaking in a constant temperature bath at 37° C for 3 hours. The samples were then removed, heated in a boiling water bath for 8 minutes, cooled in ice, filtered and adjusted to pH 7.0 with HCl and NaOH respectively. The samples were then lyophilized for assay.

Additional aliquots of fraction III_0 or portal plasma were refluxed in 0.1 N NaOH for 1 hour and in 0.1 N HCl for 1 and 2 hours. The samples were then neutralized, lyophilized and assayed unless otherwise stated in the results section.

The solution for the dialysis of portal plasma at pH 5.0 was made with 0.1 M ammonium formate buffer. This buffer was later removed by sublimation (11).

RESULTS

It has been demonstrated that the ACTH-releasing activity of portal vessel plasma was attributable to protein fraction III_0 (3). Fraction III_0 from portal plasma was further separated by moving boundary electrophoresis. Three components were evident at five different pH values, and their mobilities were determined (Table 1). Graphic representation of the electrophoretic mobilities as a function of pH showed the isoelectric points of the three components to be at pH 4.4, pH 5.1 and pH 6.6 (Fig. 1). Figure 2 illustrates an electrophoretic pattern obtained by moving boundary electrophoresis of fraction III_0 in acetate buffer at pH 3.6 ($\mu = 0.1$). A similar pattern was obtained when the proteins of III_0 were separated by starch zone electrophoresis when this same acetate buffer was used. The ACTH-releasing activity migrated in that part of the distribution which

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[2] Visking Corporation, Chicago, Illinois.

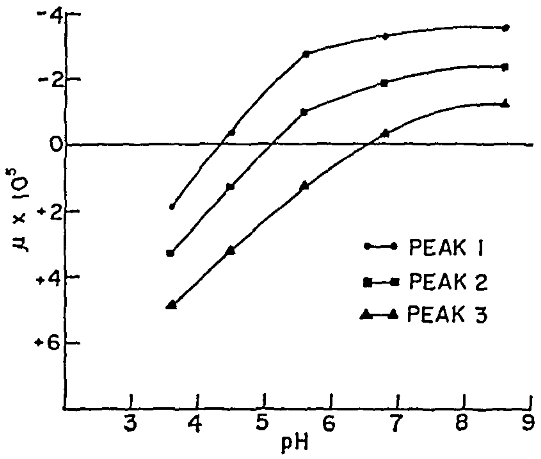

FIG. 1. Electrophoretic mobility (μ) of fraction III_0 components as a function of pH. The isoelectric points occur at the following pH values: 4.4, 5.1 and 6.6. The numbering of the peaks here is identical to that used in Table 1.

corresponded to the second of three protein peaks. The active portion is indicated by the shaded area in Figure 3 which also corresponded to the second peak observed by moving boundary electrophoresis (Table 1). The latter had a mobility of $-2.47 \pm .09$ cm.^{2} volt^{-1} sec.$^{-1} \times 10^{-5}$ in veronal buffer at pH 8.6 ($\mu = 0.1$) (Table 1) and an isoelectric point at pH 5.1 (Fig. 1). However, when the active protein peaks from several starch electrophoretic separations were pooled and then measured by moving boundary electrophoresis at pH 8.6 ($\mu = 0.1$) in veronal buffer, a single peak with a mobility of $-1.91 \pm .41$ was found. This would be compatible with the view that the active protein corresponded to a slow component of peak 2 or a fast component of peak 3 (Table 1). ACTH-releasing activity was not re-

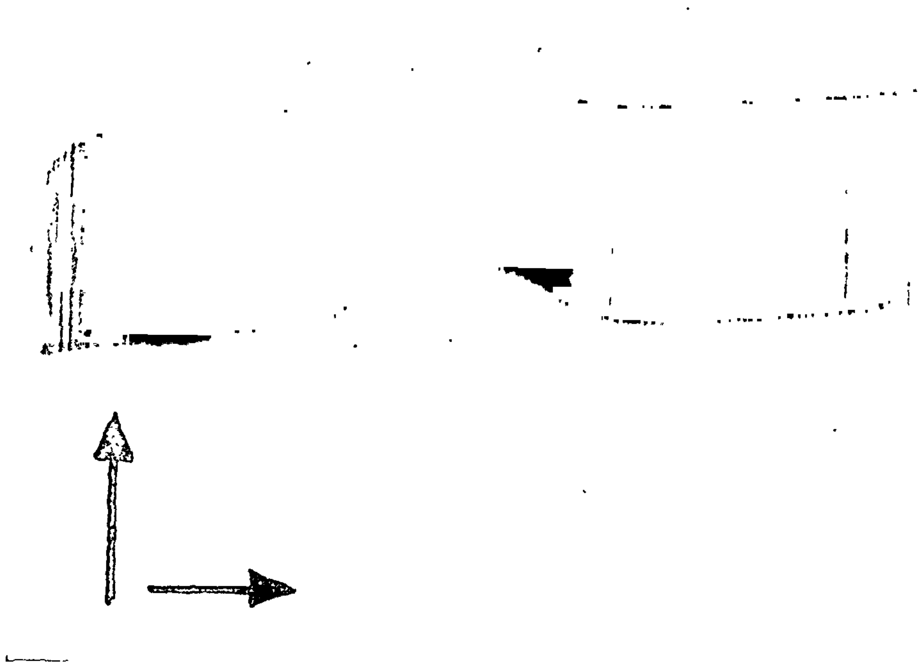

FIG. 2. Moving boundary electrophoresis of fraction III_0 in acetate buffer at pH 3.6 and 0.1 ionic strength.

TABLE 1. ELECTROPHORETIC MOBILITY OF THE PROTEIN COMPONENTS OF FRACTION III_0 FROM HYPOPHYSEAL PORTAL VESSEL PLASMA

Buffer	pH	Ionic strength (μ)	Mobility*		
			Peak 1	Peak 2	Peak 3
Veronal	8.6	0.1	−3.43 ± .13**	−2.47 ± .09	−1.25 ± .30
Phosphate	6.8	0.1	−3.23 ± .06	−1.88 ± .22	−0.28
Cacodylate	5.6	0.1	−2.71 ± .02	−0.98 ± .17	+1.29 ± .09
Acetate	4.5	0.1	−0.37 ± .06	+1.27 ± .14	+3.23 ± .13
Acetate	3.6	0.1	+1.89	+3.31 ± .47	+4.91 ± .43

* $cm.^2 volt^{-1} sec.^{-1} \times 10^{-5}$.
** Mean and standard deviation.

covered from the starch column in every case following electrophoretic separation of III_0. However, in those separations in which activity was recovered the quantity of protein in the active peak varied from 6 to 36% of the total amount of protein. The median percentage was 15.6%. The pattern chosen for illustration in Figure 3 was representative; the active peak had 12% of the total protein on the column.

The activity was destroyed by incubation with trypsin or pepsin for 3 hours and by refluxing for 1 hour with 0.1 N NaOH. Conversely, the activity of III_0 was not destroyed by refluxing with 0.1 N HCl for periods up to 2 hours duration (Table 2).

Dialysis of portal plasma against 0.1 N HCl for 25 hours or against ammonium formate at pH 5 for 24 hours did not affect the ACTH-releasing ac-

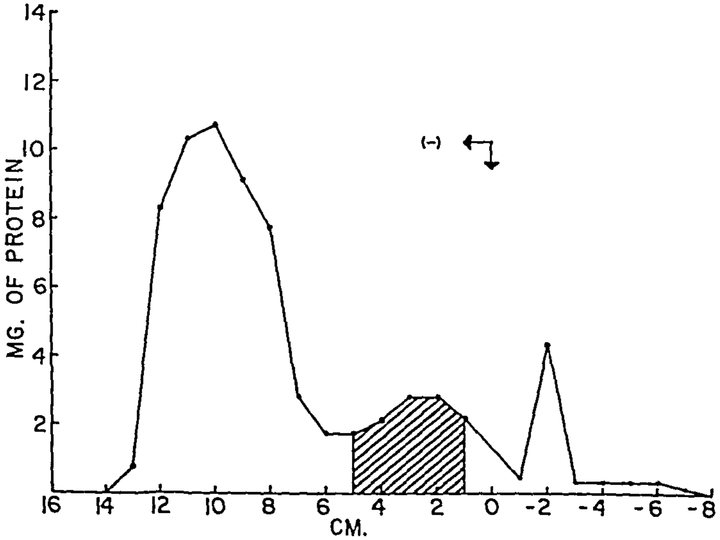

FIG. 3. Separation of the components of fraction III_0 by starch column electrophoresis in acetate buffer at pH 3.6 and 0.1 ionic strength. The column was run for 22$\frac{3}{4}$ hours at 160 volts and 18 milliamperes at a temperature of 4° C. The ACTH-releasing activity was present in that portion of the III_0 proteins illustrated by the shaded area of this pattern.

TABLE 2. EFFECT OF TRYPSIN, PEPSIN AND REFLUXING WITH NaOH AND HCl ON ACTH-RELEASING ACTIVITY OF FRACTION III_0 FROM PORTAL VESSEL PLASMA

Number of rats	Sample	Treatment	Change in adrenal ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)
2	III_0 5 cc. equivalent*	None	-51 ± 12.0**
4	III_0 10 cc. equivalent	None	-95 ± 21.1
1	III_0 10 cc. equivalent	3 hr. incubation with trypsin at pH 8.3 at 37° C	+ 5
1	III_0 10 cc. equivalent	3 hr. incubation with pepsin in 0.1 N HCl at 37° C	+ 1
1	III_0 10 cc. equivalent	Reflux 1 hr. with 25 cc. 0.1 N NaOH	+ 6
1	III_0 5 cc. equivalent	Reflux 1 hr. with 25 cc. 0.1 N HCl	−33
1	III_0 10 cc. equivalent	Reflux 2 hr. with 25 cc. 0.1 N HCl	−61

* 5 cc. and 10 cc. equivalent refers to that volume of fraction III_0 from the Cohn fractionation equivalent to 5 cc. and 10 cc. of original portal plasma, respectively. III_0 contains approximately 5 mg. protein per 1 cc. equivalent.

** Mean and standard error.

tivity; no activity was detected in the dialysate (Table 3). However, when lyophilized portal plasma was placed in the buffer solution used to dissolve fraction III_0 during plasma protein fractionation (3), activity was found outside the dialysis bag following dialysis against distilled water. Some activity was also found outside the bag when portal plasma was dialyzed for 24 hours against 15.2% (v/v) ethanol. An ethanol concentration of 15.2% (v/v) was identical with that in the buffer solution used to dissolve III_0 during plasma protein fractionation (6). When an ethanol-portal plasma mixture (78% ethanol by volume) was allowed to stand at −20° C for 48 hours, all of the ACTH-releasing activity remained with the protein precipitate. Following similar treatment of carotid artery plasma, there was no activity in either the precipitate or supernatant.

DISCUSSION

Following the observation that lyophilized hypophyseal portal plasma would elicit ACTH release in the steroid-treated rat, as measured by adrenal ascorbic acid depletion, it was found that the ACTH-releasing activity was not lost on dialysis. This activity was in protein fraction II+III (2); it was found later that the ACTH-releasing activity of portal plasma was present in a subfraction of III, *viz.* III_0 (3, 4, 5).

In the present study III_0 was observed to consist of three major proteins with isoelectric points at pH 4.4, pH 5.1 and pH 6.6. Assay of the proteins of fraction III_0 which had been separated by starch column electrophoresis showed that intermediate peak 2 (Table 1), or more likely a component of this peak, was associated with the active substance. Moving boundary electrophoresis in veronal buffer of combined active peaks from the starch column electrophoretic separations demonstrated a single component with a mobility, -1.91 ± 0.41 cm.^{2} volt.$^{-1}$ sec.$^{-1} \times 10^{-5}$, midway between that of

Table 3. Studies on the dissociation of ACTH-releasing activity from portal plasma protein

Volume of portal plasma	Treatment	Change in adrenal ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)		
		Prior to treatment	After treatment	
			Inside dialysis bag	Outside dialysis bag
15 cc.	(a) sample adjusted to pH 1 (b) dialyzed for 25 hrs. against a total volume of 1 liter of 0.1 N HCl (c) dialyzate was changed 4 times	−112*	− 98	+ 4
15 cc.	(a) refluxed with 25 cc. of 0.1 N HCl for 1½ hrs. (b) dialyzed for 6 hrs. against a total volume of 1 liter distilled water (c) dialyzate was changed 3 times	− 98	− 99	—
15 cc.	(a) sample adjusted to pH 5.0 with formic acid (b) dialyzed for 24 hrs. against a total volume of 1 liter of ammonium formate buffer at pH 5.0 (c) dialyzate changed 4 times	−112*	−142	—
15 cc.	(a) sample adjusted to pH 6.9 with the buffer used to dissolve fraction III_0 during Cohn fractionation (b) dialyzed for 24½ hrs. against a total volume of 890 cc. of distilled water. (c) dialyzate was changed 4 times	−112*	− 36 − 89	−42
10 cc.	(a) — (b) dialyzed for 24 hrs. against a total volume of 750 cc. of 15.2% (v/v) ethanol (c) dialyzate was changed 4 times	—	− 57	−25

* A 10 cc. aliquot was assayed prior to treatment.

peaks 2 and 3 (Table 1). The isoelectric point of this protein would be between pH 5.1 and pH 6.6. The amount of protein in peak 2 varied from 6 to 36% of the total protein. These observations suggested that the active constituent might be associated with a smaller amount of protein than is usually found in this peak.

During the preparation of fraction III_0 for assay it was previously found that the order in which lyophilization and dialysis were carried out affected the activity of the resulting product (3). Dialysis prior to lyophilization resulted in loss of activity; this could be interpreted as destruction of the active substance or dissociation of the active material from the protein. In this regard it was of possible significance that an active protein component was not always recoverable following starch column electrophoretic separations. The lack of success in these instances, however, was by no means apparent by inspection of the resulting protein distribution.

Destruction of the activity of III_0 occurred during incubation with trypsin and pepsin and during refluxing with 0.1 N NaOH. However, the active component was quite stable to refluxing in 0.1 N HCl. Following dialysis against distilled water for 24 hours, ACTH-releasing activity was present in the dialysate when lyophilized portal plasma was dissolved in the Cohn buffer used to fractionate III_0 (6).

This observation would support the postulate that the constituent in portal plasma responsible for ACTH release in the steroid-treated rat is dissociable from the plasma proteins. In view of these results dissociation rather than destruction of the active substance would appear to be the most likely cause for the loss of activity of III_0 previously reported (3). It is interesting to speculate that the protein component of III_0 described here functions in a transport role for a neurohumoral agent which increases the rate of release of ACTH.

Acknowledgment

The authors wish to express their thanks to Miss Elizabeth Parsons, Mrs. Julia Hallford and Mr. Tom Sullens for excellent technical assistance during the course of this investigation.

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THE ACTION OF Δ^1 HYDROCORTISONE ON THE ANTIDIURETIC AND ADRENOCORTICOTROPIC RESPONSES TO NOXIOUS STIMULI[1]

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ABSTRACT

Rats treated with large doses of Δ^1 hydrocortisone do not exhibit a decrease in the adrenal ascorbic acid concentration after exposure to painful stimuli, injection with histamine or injection with nicotine. Such animals do respond to ACTH. Hydration results in a greater diuresis in Δ^1 hydrocortisone-treated than in untreated rats. Exposure to noxious stimuli produces the same antidiuretic response in treated and untreated rats if such measures are taken in 20 or more hours after the injection of the corticosteroid. The minimal dosage of Pitressin intraperitoneally necessary to produce a significant decrease in the concentration of the adrenal ascorbic acid is more than 10 times greater in the hydrocortisone-treated than in the untreated rats. A significant decrease in adrenal ascorbic acid is produced in the treated animals by the intravenous injection of approximately twice the dosage required intraperitoneally by the untreated rat. Increasing the intravenous dosage produces no further depletion of ascorbic acid in the adrenals of treated animals. Identical adrenal cortical responses are obtained in the hydrocortisone-treated rats with similar intravenous doses of Pitressin, arginine-vasopressin and lysine-vasopressin.

The data are interpreted to indicate that whereas the treatment with Prednisolone does not affect the responsiveness of the hypothalamus, it decreases markedly the responsiveness of the adenohypophysis and neurohypophysis of animals exposed to noxious stimuli.

EXPOSURE of animal or man to noxious stimuli results in an antidiuretic response (1, 2) and a marked increase in the antidiuretic (2, 3, 4) and adrenocorticotropic (5, 6) activities of the blood. The close association between the antidiuretic and adrenocorticotropic responses to noxious stimuli led to the hypothesis that the antidiuretic hormone (ADH), vasopressin, may be responsible for the activation of the adenohypophysis and the release of adrenocorticotropic hormone (ACTH) into the circulation (2, 7). In support of this hypothesis is the rapidity with

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which ADH is released into the circulation of animals exposed to noxious stimuli (1, 2), the rapidity with which the hypothalamus is depleted of its neurosecretory material and the associated vasopressin activity (7), the relative efficacy with which exogenous vasopressin induces a release of ACTH into the circulation (8, 9, 10, 11, 12), and the inhibition of the discharge of ACTH into the circulation of animals with lesions which prevent the release of vasopressin from the hypothalamus (13). In accord also are the observations that some pharmacological agents which inhibit the pituitary-adrenal response to a variety of stimuli do not appear to impair significantly the response to Pitressin (9, 10, 11, 14).

If the secretion of ACTH is dependent upon the secretion of vasopressin, then an agent which blocks the release of ACTH might operate by inhibiting the discharge of vasopressin. Accordingly, it became pertinent to investigate the influence of treatment with such an agent on the discharge of vasopressin into the circulation. Since hydrocortisone appears to be an efficient inhibitor of the mechanism responsible for the release of adrenocorticotropin, the effect of treatment with Δ^1 hydrocortisone on the antidiuretic and adrenal ascorbic acid responses to noxious stimuli were determined.

MATERIALS AND METHODS

Male albino rats of the Carworth strain, weighing from 120 to 220 gm. were used after a preliminary fast of approximately 16 hours during which time the animals were permitted access to water. During the experiments the rats were kept in individual metabolic cages. The animals were given 5 ml. of 0.2% sodium chloride per 100 gm. body weight by stomach tube and one hour later the gavage was repeated. At 20 minute intervals after the second gavage, the volume of urine excreted was measured and all rates of excretion were expressed as ml. per 100 gm. of body weight. The animals were handled and prodded just prior to each measurement of urine volume in order to insure the emptying of their bladders. The noxious stimuli were applied at 20 minutes after the second gavage. The animals that were treated with the steroid were given a subcutaneous injection of 10 mg. Δ^1 hydrocortisone (Prednisolone®)[3] in 0.5 ml. 70% alcohol per 100 gm. body weight at 20 to 24 hours before the first gavage.

Three types of noxious stimuli were used in this study: (1) painful stimuli produced by a two-minute period of mild electric shocks (50 volts, 60,000 ohms) to the feet through a grid which replaced the floor of the metabolic cage, (2) the intraperitoneal injection of 1 mg. histamine dihydrochloride, and (3) the intraperitoneal injection of 0.1 mg. nicotine tartrate per 100 gm. body weight. The rats that served as controls for the histamine and nicotine-treated groups received an injection of an equivalent volume of normal saline. The creatinine concentration of the urine was measured by the technique described by Peters (15).

The antidiuretic activity of the plasma was assayed by a procedure which was essentially that of Jeffers *et al.* (16). In accord with the observations reported by Dettelbach (17), the rats were prepared for the assay by gavaging them three times weekly with 10 ml. of a 0.2% saline solution during at least two weeks. The day of the assay, 2 or 3 animals were selected and given 5 ml. of a 2% solution of ethanol in 0.2% saline at 37° C per 100 gm. body weight by stomach tube. One hour later, the same amount of a 10 to 12% solution of ethanol in 0.2% saline was administered. At the end of the next hour each animal was given the same volume of the 2% ethanol solution. The animal that excreted the largest volume of urine during this period was selected for the assay. The bladder of the animal was exposed by a midline suprapubic incision and a polyethylene catheter inserted and fixed in place. The load of water was kept constant at between 7 to 8% of the body weight by gavaging the rat every ten minutes with an amount of the 2% ethanol equivalent to that excreted in the previous 10 minutes. All injections were made by means of a siliconized 22-gauge needle which was inserted into one of the lateral tail veins and kept in place with ties and tape. Blood from decapitated rats was collected in plastic tubes containing approximately 5 units heparin per ml., the plasma separated rapidly and assayed within 30 minutes. A maximum amount of 0.1 ml. plasma per 100 gm. body weight was injected into the assay animal. The volume of urine excreted during the 10 minutes after an injection was expressed as the percentage of that excreted for the 10 minutes prior to the injection. A preparation of Pitressin[4] was used as a standard and the antidiuretic activity of the plasma was expressed in terms of mυ. Pitressin per 100 ml.

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[3] We are indebted to Dr. H. F. Hailman of the Upjohn Company for generous supplies of Prednisolone.

The decrease in the ascorbic acid concentration of the adrenal glands was used as a gauge of corticotropin release. The rats were decapitated one hour after exposure to the noxious stimuli, the adrenal glands removed, dissected clean and the ascorbic acid content determined by the method of Roe and Kuether (18) as modified by Sayers *et al.* (19). Animals given injections of normal saline served as controls. All animals were adapted for the assay by the daily intraperitoneal injection of 0.5 ml. saline for at least four consecutive days prior to the experiment.

RESULTS

Adrenal Ascorbic Acid Response

Twenty hours after the subcutaneous injection of Δ^1 hydrocortisone, 11 rats were exposed to the painful stimuli, 9 rats were given an intraperitoneal injection of histamine and a similar number of animals were given an injection of nicotine. Similar numbers of rats served as controls for each group and were injected intraperitoneally with saline. The data depicted in Figure 1 reveal that the dose of Δ^1 hydrocortisone used in this study completely prevented the reduction in the concentration of ascorbic acid which usually follows exposure to noxious stimuli. Whereas the concentrations (mean ±S.E.) of the adrenal ascorbic acid of the animals exposed to pain, injected with histamine and injected with nicotine was 563 ±21.6, 566 ±29.9 and 543 ±14.9 mg.%, those of the respective controls were 557 ±20.5, 554 ±30.1 and 519 ±14.6 mg.%.

To exclude the possibility that the inhibition of the response to noxious stimuli was due to an impairment in the responsiveness of the adrenal cortex, the action of adrenocorticotropin was studied. A group of 16 rats was treated with Δ^1 hydrocortisone and 20 hours later, 8 animals were each given an intraperitoneal injection of 1 mυ. adrenocorticotropin per 100 gm. of body weight and 8 were injected with saline. One hour later the adrenals

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[4] We are indebted to Dr. D. A. McGinty of the Parke, Davis and Company for generous supplies of Pitressin.

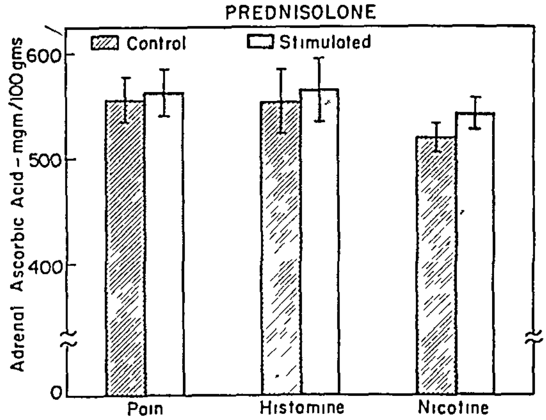


FIG. 1. Effect of Various Noxious Stimuli on the Concentration of Ascorbic Acid in Adrenals of Δ¹ Hydrocortisone-Treated Rats.

The bars depict the concentration of ascorbic acid (mean ± S.E.) in adrenals of animals exposed to the stimuli and of controls injected with saline, one hour before removal of the glands.

were removed and the ascorbic acid concentration determined. Whereas the adrenal ascorbic acid of the saline-injected rats was 474 ± 16.8 mg.%, that of the adrenocorticotropin-injected rats was 399 ± 17.8 mg. % ($P < 0.01$).

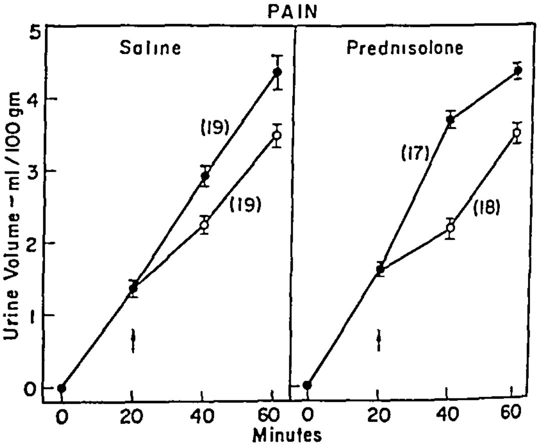
Antidiuretic Response

The rate of urine excretion (mean ± S.E.) by the saline-treated rats used as controls was fairly constant (Fig. 2). Exposure to the painful stimuli, however, resulted in a significant antidiuretic response. Whereas the rats exposed to the painful stimuli excreted 0.88 ± 0.09 ml. during the second twenty minute interval, their controls excreted 1.54 ± 0.08 ml. during the same interval ($P < 0.001$).

In accord with the observations of Gaunt *et al.* (20) who first demonstrated that corticosteroids promote the excretion of water, the administration of Δ¹ hydrocortisone resulted in an increased output of urine. Whereas rats treated with Prednisolone excreted 3.34 ± 0.08 ml., the saline-treated rats excreted 1.87 ± 0.14 ml. during the hour following the first gavage. Likewise, an increased rate of urine excretion occurred after the second gavage, especially during the first 40 minutes after the second

FIG. 2. Effect of Δ¹ Hydrocortisone on the Antidiuretic Response of Rats to Painful Stimuli.

The curves represent volume of urine excreted at each designated interval (mean ± S.E.) by saline-treated and hydrocortisone-treated rats. At the time designated by the arrow, one group of rats was exposed to the painful stimuli (○) while the other (●) was given an injection of saline. The numerals designate the number of animals in each group.



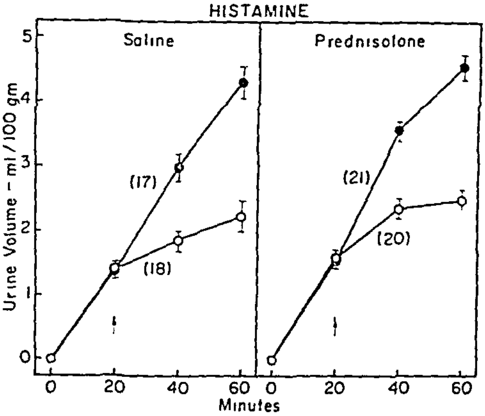


FIG. 3. Effect of Δ^1 Hydrocortisone on the Antidiuretic Response to Histamine.

The curves represent volume of urine excreted at each designated interval (mean ±S.E.) by saline-treated and hydrocortisone-treated rats. At the time designated by the arrow, one group of rats was exposed to the painful stimuli (○) while the other (●) was given an injection of saline. The numerals designate the number of animals in each group.

gavage (Fig. 2); the hydrocortisone-treated rats excreted 3.62 ±0.13 ml. and the saline-treated group excreted 2.86 ±0.13 ml. per 100 gm. body weight ($P<0.01$). Although the rate of excretion by the Prednisolone-treated rats was not constant during the three 20-minute intervals following the second gavage, a significant antidiuretic response followed exposure to the painful stimuli. Thus, the rats exposed to the noxious stimuli excreted 0.55 ±0.10 ml. during the 20-minute interval following the pain, while their controls excreted 2.09 ±0.06 ml. ($P<0.001$). The antidiuretic response of the Δ^1 hydrocortisone-treated group was even more pronounced than that of the saline-treated group.

A more intense antidiuretic response occurred after the administration of histamine and nicotine (Fig. 3 and 4). Saline-treated rats injected with histamine excreted 0.43 ±0.12 ml. during the second, and 0.39 ±0.11 ml. during the third 20-minute interval, while their respective controls excreted 1.64 ±0.11 and 1.31 ±0.11 ml. during the same intervals ($P<0.001$). Prednisolone-treated rats behaved essentially the same way. Thus, the saline-injected rats excreted 2.02 ±0.11 ml. and 0.98 ±0.13 ml. during the two consecutive periods after the injection, while the histamine-treated rats excreted 0.78 ±0.11 ml. and 0.15 ±0.04 ml. respectively ($P<0.001$).

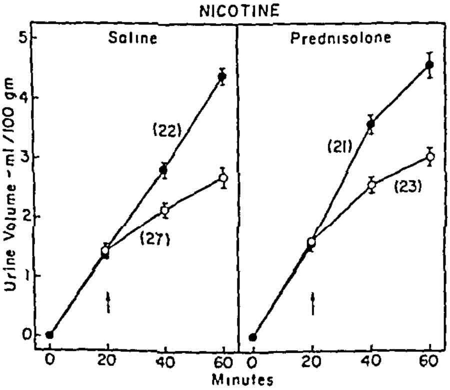


FIG. 4. Effect of Δ^1 Hydrocortisone on the Antidiuretic Response to Nicotine.

The curves represent volume of urine excreted at each designated interval (mean ±S.E.) by saline-treated and hydrocortisone-treated rats. At the time designated by the arrow, one group of rats was exposed to the painful stimuli (○) while the other (●) was given an injection of saline. The numerals designate the number of animals in each group.

The normal rats given nicotine excreted 0.73 ±0.17 ml. and their saline-injected controls excreted 1.44 ±0.07 ml. during the first 20 minutes after injection. During the third 20-minute period after the gavage, the excretion of the nicotine-injected group amounted to 0.54 ±0.08 ml. as compared to 1.56 ±0.07 ml. excreted by the control animals ($P < 0.001$). In Δ^1 hydrocortisone-treated rats these values amounted to 0.94 ±0.10 ml. as compared to 2.02 ±0.11 ml. in the second 20-minute interval and 0.46 ±0.08 ml. as compared to 0.98 ±0.13 ml. for their respective controls during the last 20-minute period ($P < 0.001$).

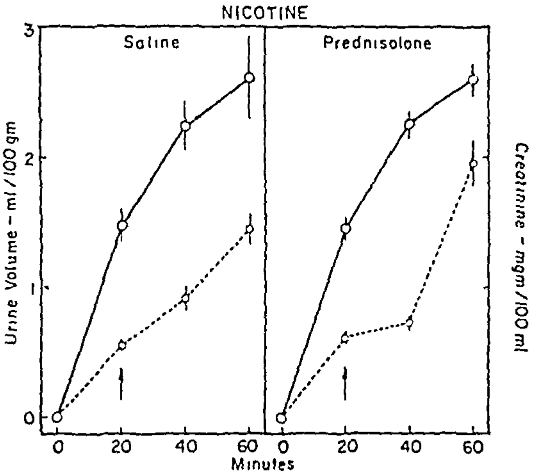


FIG. 5. Effect of Nicotine on Volume and Creatinine Concentration of Urine Excreted by Saline- and Δ^1 Hydrocortisone-Treated Rats.

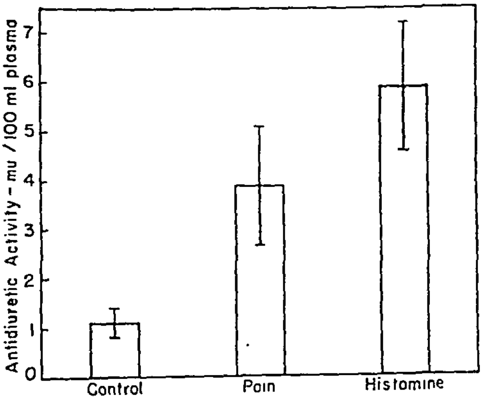
Each curve depicts the volume of urine (mean ±S.E.) (o——o) and concentration of creatinine (mean ±S.E.) (o– – – –o) at designated intervals before and after the injection of nicotine (↑).

In order to establish that the antidiuretic response of Prednisolone-treated and untreated rats was due to an increase in the reabsorption of water rather than to a decrease in glomerular filtration, the creatinine concentration of the urine before and after the injection of nicotine was measured (Fig. 5). The creatinine concentration of the urine excreted by the untreated rats during the first 20-minute interval was 5.4 ±0.4 mg.% before the injection of nicotine and rose to 9.1 ±1.0 and 14.5 ±1.2 mg.% during the following two 20-minute periods. In the Δ^1 hydrocortisone-treated rats the concentration of creatinine in the urine was 6.1 ±0.7 mg.% before the injection, and 7.8 ±0.5 mg.% and 19.6 ±1.7mg.% during the second and third 20-minute intervals after the injection. In both groups the creatinine concentration of the urine excreted during the third 20-minute period was significantly higher than that found before the injection ($P < 0.001$).

The antidiuretic activity of the plasma was assayed in order to determine its relation to the antidiuretic response of rats as measured by the decrease in urine excretion. The activity of the plasma (mean ±S.E.) of normal rats was equivalent to 1.1 ±0.33 mu. Pitressin per 100 ml. (Fig. 6). Thirty seconds after the rats were exposed to the painful stimuli the antidiuretic activity was equivalent to 3.9 ±1.2 mu. per 100 ml. plasma. A more pronounced increase in the antidiuretic activity of the plasma

Fig. 6. Effect of Painful Stimuli and of Histamine on the Antidiuretic Activity of the Plasma.

Each bar represents the antidiuretic activity of the plasma of 16 rats (mean ± S.E.) expressed in terms of Pitressin (see text).



(5.9 ±1.3 mu. per 100 ml.) was found in 10 minutes after the intraperitoneal injection of histamine ($P < 0.01$).

Response to Pitressin

The effect of the *intraperitoneal* injection of various doses of Pitressin on the concentration of adrenal ascorbic acid in saline and Δ^1 hydrocortisone-treated rats is detailed in Table 1 and illustrated in Figure 7. It is evident that a significant reduction in the adrenal ascorbic acid concentration of saline-treated rats followed the injection of 0.1 or more units of Pitressin per 100 gm. body weight. In accord with general experience the initial concentration of ascorbic acid in the adrenals of the rats treated

Table 1. Effect of pitressin on the ascorbic acid concentration of the adrenal glands of Δ^1 hydrocortisone-treated and untreated rats

The Pitressin was administered intraperitoneally (a) or intravenously (b) and the adrenals were removed one hour later for assay. The numerals in parentheses refer to the number of animals.

Pitressin units per 100 gm.	Adrenal ascorbic acid—mg. per 100 gm.		
	Controls	Prednisolone	
	a	a	b
0	394 ± 9.3 (26)	—	—
0.02	368 ±14.5 (27)	—	—
0	393 ±10.4 (23)	—	516 ±22.7 (15)
0.1	312 ±12.7 (22)***	—	490 ±26.4 (15)
0	397 ±11.7 (18)***	479 ±17.7 (10)	521 ±11.0 (16)**
0.2	273 ± 9.8 (19)	495 ±20.0 (10)	451 ±13.2 (16)
0		479 ±17.7 (10)	477 ±11.5 (15)**
0.5		488 ±14.6 (10)	399 ±12.5 (15)
0	414 ±16.9 (10)***	431 ±14.7 (15)*	505 ±16.9 (9)*
1.25	233 ±16.1 (10)	361 ±24.2 (15)	422 ±20.0 (10)

* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0.1% level.

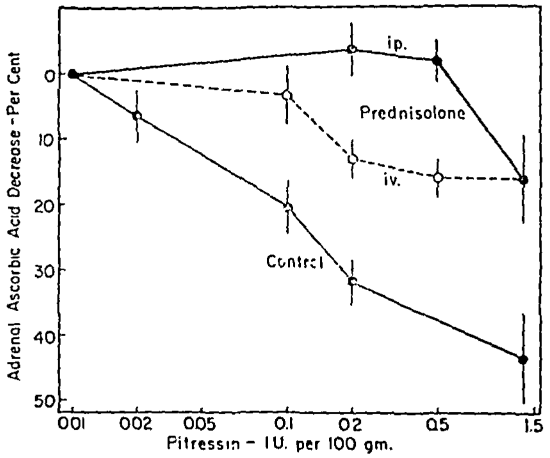


FIG. 7. Effect of Pitressin on the Adrenal Ascorbic Acid Δ¹ *Hydrocortisone-Treated* Rats.

The change in adrenal ascorbic acid concentration expressed as the per cent (mean ± S.E.) of values found in controls.

with Prednisolone was much higher than that found in untreated rats. Yet, the Δ¹ hydrocortisone-treated rats did not show a significant change in the adrenal ascorbic acid concentration until a dose of 1.25 units Pitressin per 100 gm. body weight was administered. Thus, more than ten times the dosage of Pitressin was necessary to produce the same adrenal ascorbic acid depletion in the Δ¹ hydrocortisone-treated rats as in the saline-treated controls.

The *intravenous* injection of 0.2 unit Pitressin per 100 gm. body weight was effective in reducing the concentration of ascorbic acid in the adrenals of the Prednisolone-treated rats. Increasing the dosage to as high as 1.25 u. per 100 gm., however, did not produce a further depletion of the ascorbic acid (Fig. 7).

That the adrenal ascorbic acid response to the intravenous injections to Pitressin is probably not due to some contaminant is suggested by the effects of purified arginine-vasopressin and synthetic lysine-vasopressin.[5] Three groups of 16 rats were treated with Δ¹ hydrocortisone and 20 hours later, half of each group was given an intravenous injection of 0.2 pressor unit of one of the antidiuretic compounds per 100 gm. of body weight while the other half of each group was injected with saline. One hour later the adrenals were removed and the ascorbic acid concentration determined. The data (mean ±S.E.) depicted in Figure 8 reveal that the three compounds produced essentially the same depletion in the adrenal ascorbic acid.

DISCUSSION

In accord with previous studies (2, 21), the experiments described herein reveal that exposure to various noxious stimuli such as pain, the injection of histamine and the injection of nicotine results in an antidiuretic response in normal rats. The concomitant increase in the concentration of creatinine in the urine indicates that the antidiuretic response is due to an increase in the rate of water reabsorption by the renal tubules rather than to a decrease in glomerular filtration. The concomitant increase in the antidiuretic activity of the plasma suggests that the increased reabsorption of water by the renal tubules is due to the discharge of vasopressin into the circulation.

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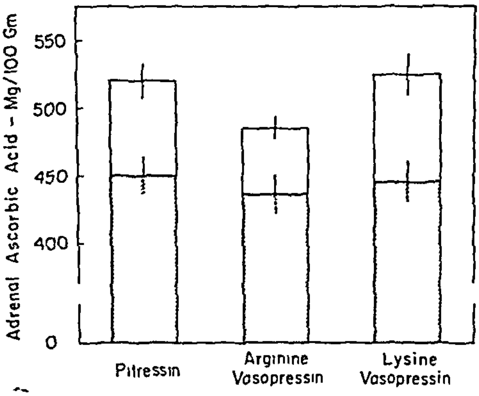
[5] We are indebted to Dr. Vincent du Vigneaud for generous supplies of compounds.

The antidiuretic response which occurs after exposure of an animal to a noxious stimulus may be due to the discharge of the antidiuretic hormone (ADH) directly from the hypothalamus into the general circulation and/or the discharge of ADH from the neurohypophysis (1, 2, 7, 21, 22, 23, 24, 25, 26). The essentially identical antidiuretic responses of the Δ^1 hydrocortisone-treated and saline-treated rats suggest that the steroid did not affect the functional integrity of the hypothalamus. A block to the discharge of ADH from the neurohypophysis, however, is not precluded by these data. That Δ^1 hydrocortisone may induce an inhibition of the mechanism responsible for the discharge of ADH from the neurohypophysis without affecting that of the hypothalamus is suggested by the observations of Gaunt *et al.* (25). They found that the injection of formalin resulted in a marked reduction in the concentration of ADH in the hypothalamus of both normal and hydrocortisone-treated rats. The same insult resulted in a decrease in the concentration of ADH in the neurohypophysis of normal rats and an increase in that of the hydrocortisone-treated rats. Such an inhibition of the mechanisms responsible for the discharge of ADH from the neurohypophysis may account for the greater diuresis which occurs after hydration in animals treated with hydrocortisone than in controls.

FIG. 8. Effect of Intravenous Injection of Pitressin, Arginine-Vasopressin and Lysine-Vasopressin on Adrenal Ascorbic Acid of Δ^1 Hydrocortisone-Treated Rats. Each group consisted of 16 rats treated with hydrocortisone. Half of each group was injected with saline (open bars) and half with the designated compound (cross-hatched bars). One hour later the adrenals were removed and the ascorbic acid concentration (mean ± S.E.) determined.



The antidiuretic responses of the Δ^1 hydrocortisone-treated rats are not in complete agreement with the data reported recently by McCann *et al.* (27) who found that four hours after the intraperitoneal administration of 8 mg. hydrocortisone acetate per 100 gm. body weight, rats develop only about one half of the antidiuretic response observed in normal animals exposed to noxious stimuli. The discrepancy, however, is due to the fact that whereas McCann *et al.* performed their experiments in four hours after the injection of the hydrocortisone, the data reported herein were collected in 20 to 24 hours after the administration of Prednisolone. Thus, when a group of 10 rats were hydrated and given nicotine four hours after the subcutaneous injection of Δ^1 hydrocortisone, they developed an antidiuretic response which was only 43 per cent of that exhibited by a group of 10 saline-treated controls. A similar decrease in the antidiuretic response occurred when rats were given nicotine 2 hours after the injection of the Δ^1 hydrocortisone. When the same groups were retested 25 hours after the initial subcutaneous injections, however, identical antidiuretic responses to nicotine were obtained in both the Δ^1 hydrocortisone and saline-treated groups.

Although McCann *et al.* (27) attribute the partial inhibition of the antidiuretic response to noxious stimuli to an inhibition of the hypothalamus, it is quite possible that other mechanisms may be involved. Thus, the immediate response may reflect an inhibition of the peripheral action of vasopressin such as was demonstrated by Gaunt *et al.* (20, 25). Likewise, the marked diuresis which occurs immediately after the injection of the corticosteroids may mask the antidiuretic response to noxious stimuli. It was in order to obviate the effects of these and other factors that the present studies were performed 20 to 24 hours after the injection of the Δ^1 hydrocortisone by which time there is no impairment in the antidiuretic response.

The decrease in the concentration of adrenal ascorbic acid which ordinarily follows exposure to noxious stimuli does not occur in the Δ^1 hydrocortisone-treated rats. Since the adrenal glands of animals treated with hydrocortisone respond to the administration of adrenocorticotropin, it is probably that the lack of response in noxious circumstances is due to an inhibition of the mechanisms responsible for the discharge of adrenocorticotropin into the circulation. Such an inhibition may be due to (a) a pharmacologically induced block in the hypothalamus similar to that which occurs with lesions in the median eminence (10, 11, 12, 13, 27, 28, 29) or (b) an inhibition of the production and/or release of adrenocorticotropin from the adenohypophysis.

That a decrease in the responsiveness of the mechanisms responsible for the release of corticotropin from the adenohypophysis can account for the effects of Δ^1 hydrocortisone is indicated by the difference in the responses of treated and untreated rats to different doses of Pitressin. Whereas a significant decrease in the concentration of the adrenal ascorbic acid of normal rats follows the *intraperitoneal* injection of 0.1 unit Pitressin per 100 gm. body weight, more than 10 times this dosage (1.25 U. per 100 gm. body weight) is required for a similar response in Δ^1 hydrocortisone-treated animals. Likewise, although the *intravenous* injection of 0.2 U. Pitressin per 100 gm. body weight does produce a significant decrease in the adrenal ascorbic acid of hydrocortisone-treated animals, increasing the dosage to 1.25 U. per 100 gm. does not result in a further significant change. Such data support the probability that the absence of an adrenal cortical response to noxious stimuli in Δ^1 hydrocortisone-treated rats is due to a decrease in the responsiveness of the ACTH releasing mechanism rather than to an inhibition in the production of the hypothetical corticotropin releasing factor by the hypothalamus.

It would appear from the preceding that the corticosteroids act directly on the pituitary gland to reduce the responsiveness of the mechanisms responsible for the release of ACTH from the anterior lobe and ADH from the posterior lobe. This hypothesis is in accord with the studies of Rose and Nelson (30) on the suppressive effect of the injection of hydrocortisone directly into the fossa hypophysis on the release of ACTH. In accord also is the adrenal cortical atrophy that occurs in corticosteroid-treated dogs (28, 29) and rats (27) with the hypothalamic lesions that prevent the usual adrenal cortical responses to noxious stimuli. Accordingly, whereas the response of the normal animal to noxious stimuli results from the stimulation of both the hypothalamus and the hypophysis, the response of the Δ^1 hydrocortisone-treated animal may be due primarily to stimulation of hypothalamic mechanisms.

The nature of the neurohormonal agent (or agents) responsible for the activation of the release of ACTH from the adenohypophysis is unknown. As indicated at the outset, a good deal of evidence can be marshalled in support of the hypothesis that vasopressin or a closely related polypeptide is the specific corticotropin releasing agent. This hypothesis appears to be supported by the identical adrenal cortical responses of Δ^1 hydrocortisone-treated rats to the intravenous injections of similar doses of Pitressin, arginine-vasopressin and lysine-vasopressin. However, the evidence that the steroid produces a marked impairment in the responsiveness of the adenohypophysis makes it impossible to utilize the present study to either support or negate the postulate that vasopressin plays a prepotent role in the release of ACTH in the animal exposed to noxious stimuli.

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CARDIAC GLYCOGEN AND THE EFFECT OF GROWTH HORMONE AMONG INBRED STRAINS OF MICE[1]

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ABSTRACT

In an effort to detect differences in the peripheral activity of growth hormone among C_{57}, A, C_3H, and I strain mice, the concentrations of cardiac glycogen were determined during the course of a 24-hour fasting period, and in three different age groups (young, 40–50 days; young-adult, 3–5 months; and old, 8–13 months). The concentrations and changes in cardiac glycogen were found to be characteristic of the age, and of the strain of mice. For example, in young-adult, but not young, mice of the I strain, cardiac glycogen actually decreased during a 24-hour fast. Some strain differences were found, but these were not consistent in all age groups. It was concluded that cardiac glycogen was subject to a variety of influences, which in turn varied with the age of the animal.

As found for the rat, exogenous growth hormone doubled the stores of cardiac glycogen but the hormone had to be administered in the morning to nonfasted mice. If it was administered in the afternoon, it was not effective. However, if insulin was given either before or with the growth hormone in the afternoon, an effect usually seen with administration of growth hormone in the morning could be duplicated. Insulin alone had no effect. This, and other evidence, suggests that insulin is required for the action of growth hormone on cardiac glycogen deposition.

STUDIES of the lipid (1), protein (2), and carbohydrate (3) metabolism of four highly inbred strains of mice have demonstrated that each strain possesses a distinctive metabolic pattern. In attempts to account for these metabolic patterns by hormonal differences, it was tentatively postulated that one of the strains, the I, has a high peripheral activity of insulin (3, 4, 5, 6). However, the possibility that the unusual carbohydrate metabolism in this strain was due to other hormonal influences could not be overlooked. Since the large stores of glycogen in the abdominal and gastrocnemius muscles of the I strain do not deplete as readily during a fast as the smaller stores in the musculature of the C_{57}, A or C_3H strains (3), the presence of larger amounts of a "carbohydrate sparing factor," such as growth hormone, in the I strain seemed possible. However, higher quantities of

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growth hormone in the other strains seemed equally possible, for the endogenous nitrogen excretion of the A strain is less (7), and the growth rate of the A or the C_3H strain is greater (2) than that of the I strain. One feasible experimental approach to this question was the determination of the stores of cardiac glycogen in these strains. Russell and her collaborators have demonstrated that the presence of growth hormone is necessary for the increase in cardiac glycogen which occurs during a fast (8, 9), and that increases in cardiac glycogen following the administration of growth hormone are directly related to the dose of the hormone (10). Moreover, the action of growth hormone on cardiac glycogen seems to be independent of insulin (8, 9).

METHODS

Animals. Mice of the A/FnLn, C_{57}BL/FnLn, I/FnLn and C_3H strains from our colony were used in these experiments. Groups of mice were housed in plastic cages and were maintained on a commercial stock ration (Purina Laboratory Chow). Except in the first group (Section A), all of the mice were males, 3–5 months of age. Food and water were supplied *ad libitum*, and during fasting periods, water was supplied *ad libitum*. The mice were anesthetized with pentobarbitol-sodium (Nembutal, Abbot), 0.1 mg. per gm. body weight.

Removal of the heart. In preliminary studies, the concentration of glycogen found in the mouse heart was considerably lower than that reported for the rat heart, although the concentrations of glycogen in the gastrocnemius or diaphragm muscles are similar in the two species (3, 9). Considering the greater metabolic and heart rate of the mouse, it was possible that glycogenolysis occurred to a greater extent in the process of removing the mouse heart. It has been shown that glycogenolysis occurs in the rat heart when the heart is beating in the opened chest (11). A simple technique was evolved for a rapid removal. The skin and fur over the rib cage were laid back. The xiphoid process was grasped with a pair of curved forceps, and the rib cage was raised slightly to facilitate a single incision through it with a pair of large scissors. The heart was removed with the curved forceps, and pressed between two blocks of dry ice. The interval from the time that the chest cavity was opened to the time when the heart was placed on the dry ice was measured with a stopwatch. For intervals from 1.8 to 7.5 seconds, no differences were evident in the concentrations of glycogen within each strain. The removal time of the heart in these studies ranged from 2 to 5 seconds. While still frozen, the heart was scraped clean, and then weighed to the nearest 0.1 mg. on a torsion balance. The other tissues were removed after the heart and in the order: liver, gastrocnemius muscle.

Analytical procedures. The methods for glycogen determination have been described (6). The results are expressed as mg. of glucose per gm. of wet weight of tissue.

Growth hormone. The purified bovine growth hormone used in these studies was prepared in this laboratory by the method of Wilhelmi, Fishman and Russell (12). Slightly alkaline, saline solutions were prepared the day that they were used. All injections were made intraperitoneally (as a single injection) in volumes of 0.05 to 0.25 ml. at a dose of 2 mg. per 100 gm. of body weight.

Insulin. The insulin used in these studies was a commercial preparation, Iletin, Eli Lilly and Company. It was given intraperitoneally in a single dose, 0.1 U. per kg. of body weight, in volumes of 0.15 to 0.25 ml.

Glucose. This was given intraperitoneally in a single dose, 3 mg. per gm. of body weight, in volumes of 0.15 to 0.25 ml.

RESULTS

A. *Studies with young and old mice.* In preliminary studies it became apparent that the levels of cardiac glycogen varied with the age of the animal. A series of observations on male and female mice of the A, C$_3$H, and I strains established that cardiac glycogen did not increase during a fast if the animals were over 8 months of age (Table 1). In fact, a slight decrease was observed in all cases, but the differences were not significant. The values among males and females, and among the three strains were remarkably similar (Table 1).

In young mice (40–50 days of age), cardiac glycogen did increase during a fast (Table 1). At this age, the early rapid growth phase is nearly completed. The fact that increases in cardiac glycogen did occur in young and not in older mice may be related to the consistently lower levels in the fed state among the young mice; the levels in the fasted state were nearly the same in the two age groups. Greater increments were found in mice of the C$_{57}$ strain (76%) than in mice of the I (38%) or A (31%) strains. Of the three strains, the A exhibits the highest growth rate (2).

TABLE 1. THE EFFECT OF FASTING ON CARDIAC GLYCOGEN IN OLD AND YOUNG MICE

Mice		Non-fasted		Fasted#	
Strain	Sex	Body wt., gm.	Glycogen‡	Body wt., gm.	Glycogen‡
		8–13 month old mice			
A	male	29.8	2.5 ±0.14 (6)*	25.3	2.3 ±0.06 (7)
C$_3$H	male	35.7	2.6 ±0.24 (5)	30.4	2.3 ±0.18 (5)
C$_3$H	female	34.5	2.5 ±0.15 (7)	31.6	2.3 ±0.17 (7)
I	male	27.8	2.4 ±0.30 (6)	24.0	2.0 ±0.25 (6)
I	female	29.6	2.6 ±0.15 (8)	27.3	2.3 ±0.13 (5)
		40–50 day old mice			
A	male	20.3	1.6 ±0.22 (7)	16.5	2.1 ±0.10† (7)
C$_{57}$	male	20.5	1.5 ±0.12 (8)	15.3	2.7 ±0.13† (9)
I	male	16.7	1.8 ±0.17 (6)	12.4	2.5 ±0.12** (8)

‡ Glycogen, mg./gm. of wet tissue weight and standard error.
* Number of mice.
# Twenty-four hour fast.
† P value for the differences between the fed and fasted mice, <0.01.
** P value for the difference between the fed and fasted mice, 0.06.

B. *Studies with young-adult mice.* In young-adult mice (3–5 months of age) of the A and C_{57} strains increases in cardiac glycogen also occurred during a fast. The changes in cardiac glycogen during the fasting period for C_{57} strain mice are plotted against time in Figure 1. In the first 12 hours of the fasting period only modest changes were evident, but after this period of quiescence, the concentration of cardiac glycogen increased sharply. The changes in cardiac glycogen of the A strain were similar to those of the C_{57} strain, and for clarity they are not shown in Figure 1. Over the 24-hour fasting period, cardiac glycogen increased in A strain mice from 1.5 ±0.12 (S.E.) mg./gm., 10 mice, to 2.2 ±0.14 (S.E.) mg./gm., 6 mice, and the increase was significant (P <0.01). The increment, about 50%, found in these two strains is comparable to that reported for the rat (9), although the absolute concentrations of glycogen are lower in the mouse.

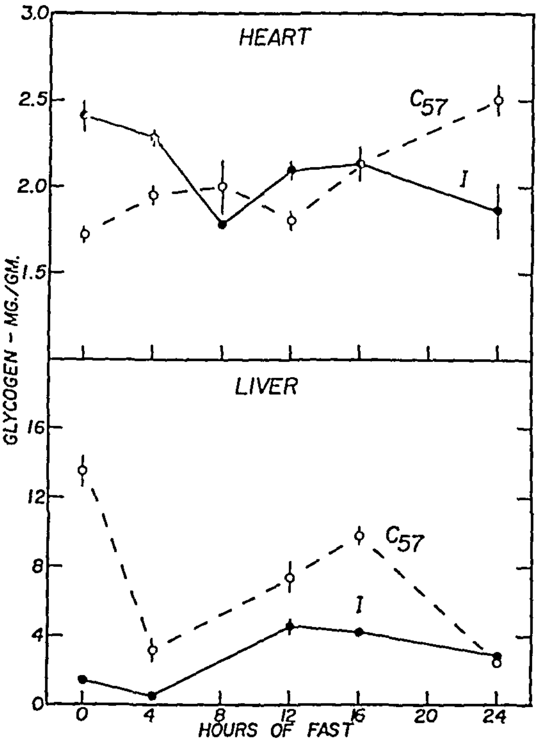

FIG. 1. Effect of fasting on cardiac and liver glycogen of C_{57} and I strain mice. The vertical bars represent the standard errors of the means of 5–16 determinations for the hearts, or 5–10 determinations for the livers. The fast began about 4:30 P.M.

In contrast to the A and C_{57} strains, a significant (P <0.01) depletion in cardiac glycogen was found in the I strain after 8 hours of fasting, and as the fast continued no further changes occurred (Fig. 1). The concentration of glycogen in the heart of non-fasted I strain mice was greater (P <0.01) than that in A or C_{57} strain mice.

The marked differences in the concentrations of liver glycogen in the I and the C_{57} strains during the experimental period (Fig. 1) suggested that physiological differences, possibly related to liver glycogen, might account for the behavior of cardiac glycogen in the I strain. At the beginning of the fast, the stores of liver glycogen were greater in the C_{57} strain, and during the latter part of the fasting period, the increases were greater in the C_{57} than in the I strain (Fig. 1). In order to elevate liver glycogen in the I strain, the feeding time was altered from the evening to the daylight hours just prior to the beginning of the fast. Mice of the I and C_{57} strains were fasted for 24 hours, and then fed for 8–9 hours. At the end of this feeding period, about 4:30 P.M., the second 24-hour fasting period was initiated. The levels of cardiac and liver glycogen during this second fasting period are given in Figure 2. The initial concentrations of liver glycogen were 29 times greater in the I strain and 6 times greater in the C_{57} strain than when the mice were permitted to eat *ad libitum* (Fig. 1). The initial concentrations of glycogen in the hearts of the two strains were also greater ($P < 0.01$) when the animals were forced to eat during the day (Fig. 2). Nevertheless, significant ($P < 0.01$) decrements in cardiac glycogen were evident in both strains within 8 hours of fasting (Fig. 2). The rate of decline in the I strain was even greater when the initial levels of cardiac glycogen were higher (Figs. 1 and 2).

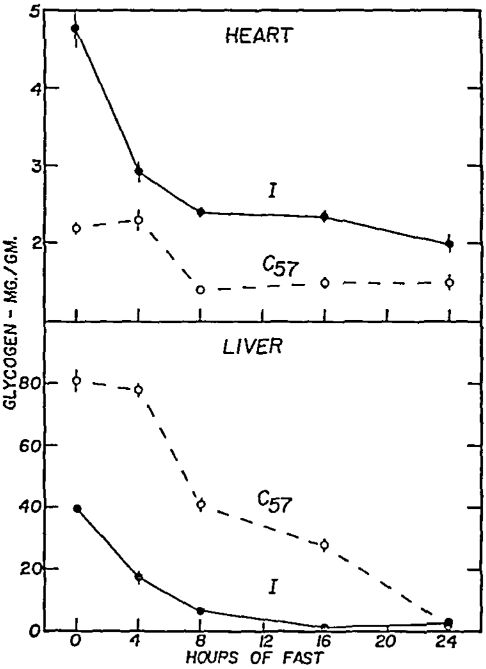

FIG. 2. Effect of fasting on cardiac and liver glycogen of C_{57} and I strain mice after preliminary fasting and feeding periods. These mice were first fasted for 24 hours, then fed for 9 hours before the beginning of the second fasting period at 4:30 P.M. The vertical bars represent the standard errors of the means of 5–8 determinations.

C. *Studies with homogenates of mouse pituitary gland.* Utilizing the changes in cardiac glycogen as an assay, an attempt was made to detect strain differences in the stores of growth hormone. Pituitary glands were removed from C_{57}, C_3H and I strain mice under nembutal anesthesia. Glands from each strain were homogenized in ice-cold saline; the final pH of the homogenate was 7.9. Homogenates from each strain, containing about one mouse pituitary gland in a volume of 0.2 ml., were administered intraperitoneally to non-fasted, young-adult C_{57} strain mice at about 9:00 A.M. After a fast of 6 hours, the concentrations of glycogen in the heart, the liver and the gastrocnemius muscle were 1.4 to 2.4 times greater than in untreated mice fasted for the same length of time (Table 2). The pituitary homogenates from each strain evoked similar increases.

D. *Studies with purified growth hormone.* The increases in cardiac glycogen found after the administration of homogenates of mouse pituitary glands (Table 2), were also found after the administration of purified preparations (12) of bovine growth hormone. In this study (Fig. 3), the hormone was given in a single dose (0.2 mg. per 10 gm. of body weight) to non-fasted mice of the C_{57} strain at about 9:00 A.M., and the mice were fasted for periods up to 6 hours. Within 3 hours the stores of glycogen in the heart were doubled, whereas only minimal changes occurred in the gastrocnemius muscle or the liver (Fig. 3). It is interesting that this dose of purified bovine growth hormone (0.4–0.5 mg.) was no more effective on the stores of cardiac glycogen than a single mouse pituitary gland, wet weight 1–2 mg. (Table 2 and Fig. 3).

TABLE 2. THE EFFECT OF HOMOGENIZED MOUSE PITUITARY GLANDS ON MUSCLE GLYCOGEN LEVELS OF C_{57} STRAIN MICE

Donor mice		C_{57} strain recipients		
		Glycogen, mg./gm.		
Strain	Body wt., gm.	Heart	Gastrocnemius	Liver
C_{57}	25.2	2.9*	4.6*	25.0*
C_3H	30.0	2.6	4.3	38.8
I	22.8	2.8	3.9	49.7
Total Average		2.8 ± 0.42#	4.3 ± 0.30	37.8 ± 5.67
Untreated C_{57} strain mice		1.6 ± 0.11 (9)†	3.1 ± 0.15 (9)	15.8 ± 4.28 (9)
P**		<0.01	<0.01	<0.01

* Each value is the mean of two determinations.
# Standard error of the mean.
† Number of mice.
** P value for the difference between the injected and the non-injected mice.

In another series of experiments, growth hormone was given to non-fasted mice of the C_{57} strain at about 4:30 P.M. After a fasting period of 4 hours, the stores of cardiac glycogen were no greater than those in untreated mice (Table 3, Part A). This failure in the activity of the hormone was puzzling, since dramatic effects were seen if the hormone was given only 7 hours earlier in the day (Fig. 3).

One possible explanation for this failure was the obvious difference in the physiological states of the mice at the two times of the day. Mice eat very little during the day, and the stores of liver glycogen deplete rapidly from 9:00 A.M. to 4:30 P.M. (Figs. 1 and 3). In order to mimic the physiological state at 9:00 A.M., mice were given intraperitoneal injections of glucose (Table 3, Part B), or were forced to eat during the day (Table 3, Part C), before growth hormone was administered at 4:30 P.M. The maximum effect of either treatment was a slight increase (20%) in activity of the hormone (Table 3, B-3 and C-2). However, if the same dose of glucose was superimposed upon the forced feeding regime, then the effect of the hormone seen after dosage at 9:00 A.M. could be duplicated (Table 3, C-3). This same response could be elicited if insulin was given prior to or simultaneously with growth hormone at 4:30 P.M. (Table 3, D-2). Insulin given after growth hormone was less effective (Table 3, D-3), and insulin alone had no effect (Table 3, D-1).

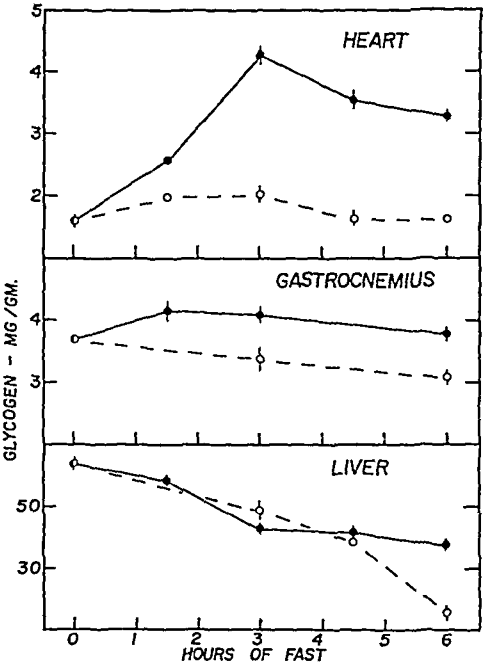

FIG. 3. Effect of growth hormone on muscle and liver glycogen of C_{57} strain mice. The solid lines represent mice injected with growth hormone (0.2 mg. per 10 gm. of body weight) at 9:00 A.M. and then fasted. The dashed lines represent fasted, untreated mice. The vertical bars represent the standard errors of the means of 5–10 determinations per point.

In the young-adult I strain mouse, cardiac glycogen decreased during a fast (Figs. 1 and 2). Attempts were made to reverse this depletion by administering purified growth hormone. Ten young-adult I strain mice were given a dose of growth hormone (0.2 mg. per 10 gm. of body weight) at 4:30 P.M., and fasted for 4 hours. The average concentration of cardiac glycogen was 2.4 ± 0.18 (S.E.) mg./gm., which was no different from that found in untreated mice (Fig. 1), or mice receiving injections of saline. A second group of 10 I strain mice were fasted for 24 hours, then fed for 8–9 hours before receiving the same dose of growth hormone at 4:30 P.M. After a 4 hour fast, the average concentration of cardiac glycogen was 3.9 ± 0.23 (S.E.) mg./gm. This value is about midway between the initial level and that found after a 4 hour fast of untreated mice forced to eat during the day (Fig. 2).

TABLE 3. THE EFFECT OF GROWTH HORMONE ON CARDIAC GLYCOGEN IN C_{57} STRAIN MICE. GROWTH HORMONE WAS GIVEN AT 4:30 P.M. TO THOSE GROUPS MARKED WITH AN (*), AND IN ALL CASES THE MICE WERE FASTED FROM 4:30 TO 8:30 P.M.

Experimental conditions	No. of mice	Glycogen		P_1	P_2
		Heart	Liver		
		mg./gm. of tissue			
A. The effect of growth hormone (G.H.)					
1. No treatment	9	2.0 ±0.12#	3.2 ±1.4	—	—
(*) 2. Growth hormone	12	2.4 ±0.15	5.6 ±1.9	0.07	—
B. The effect of a dose of glucose given prior to G.H.					
1. Glucose only, 4 hrs. before fasting	5	2.2 ±0.15	—	0.5	—
(*) 2. Glucose, 1–3 hrs. before G.H.	6	2.5 ±0.24	7.0 ±2.2	0.04	—
(*) 3. Glucose, 4 hrs. before G.H.	6	2.9 ±0.14	5.2 ±1.2	<0.01	0.04
C. The effect of an increased alimentation† prior to G.H.					
1. No treatment	6	2.3 ±0.26	77.6 ±4.7	0.19	—
(*) 2. G.H.	6	2.8 ±0.15	80.4 ±6.3	<0.01	0.07
(*) 3. Glucose, 4 hrs. before G.H.	6	3.5 ±0.44	71.9 ±3.9	<0.01	<0.01
4. Glucose, 4 hrs. before fasting	6	2.3 ±0.26	69.7 ±6.4	0.14	—
D. The effect of insulin given before, with or after G.H.					
1. Insulin only	5	1.7 ±0.17	—	—	—
(*) 2. Insulin, 1 hr. before or simultaneously with G.H.	6	3.9 ±0.17	—	<0.01	<0.01
(*) 3. Insulin, 1 hr. after G.H.	3	3.1 ±0.09	—	<0.01	0.05

# Standard error of the mean.

P_1, P value for differences in cardiac glycogen from A-1.

P_2, P value for differences in cardiac glycogen from A-2.

† All mice in this group were fasted for 24 hours, then fed for 8–9 hours before the beginning of the second fast at 4:30 P.M.

We have recently reported (13) that the I strain mouse exhibits decreasing stores of vitamin B_6 with age. Therefore, aseries of I and C_{57} strain mice were fed the stock ration supplemented with 50 mg. of pyridoxine hydrochloride per kg. of diet for 2 weeks. The concentrations of cardiac glycogen in the two strains during a 24-hour fasting period were no different from those shown in Figure 1.

DISCUSSION

If differences in the peripheral activity of growth hormone do exist in these strains of mice, they are certainly not discernible from the changes in cardiac glycogen. For example, the increments in cardiac glycogen during a fast were greater in young C_{57} than in young A strain mice (Table 1), but in young-adult mice the increments were similar in the two strains (see section B). If the increments in cardiac glycogen during a fast are solely the reflection of the titers of growth hormone, then it is difficult to explain the well-established observation that A strain mice grow more rapidly than C_{57} strain mice (2). The behavior of cardiac glycogen during a fast in the I strain presents an even more extreme picture; sizeable increases were observed in young mice (Table 1), but decreases were observed in young-adult mice (Figs. 1 and 2), and no changes were observed in older mice, as found with the other strains (Table 1).

The decrease in cardiac glycogen in the fasting young-adult I strain mouse does not seem to be due to a deficiency in the pituitary stores of growth hormone (Table 2), nor did the administration of rather large doses of bovine growth hormone appreciably alter the depletion of cardiac glycogen during a fast (see section D). In view of the fact that the I strain mouse has an unusual requirement for pyridoxine (13), it was surprising that large supplements of pyridoxine (50 mg. per kg. of diet), did not alter the behavior of cardiac glycogen (Fig. 1). However, experiments now in progress suggest that the time of feeding of the pyridoxine supplements was not optimal, and this point is still under investigation. Since Cori and Illingworth (14) have demonstrated that pyridoxal phosphate is the coenzyme of phosphorylase, and since it has been found that I strain mice fed a complete ration exhibit lower levels of vitamin B_6 at 100 than at 60 days of age (13), an explanation of the unique decrease in cardiac glycogen during a fast in terms of the vitamin B_6 metabolism offers some promise. It is evident that the large stores of muscle glycogen found in the I strain (3, 4), and their maintenance during a fast, cannot be due to an excessive output of growth hormone.

The data presented in Figures 1 and 2 and in Tables 1 and 2 demonstrate that the concentrations and the changes in cardiac glycogen in the mouse must be related to the age of the animal. Secondly, the stores of cardiac glycogen must be subject to a variety of controls which in turn vary with the age and the strain of the mouse.

One of these controls would appear to be a requirement for the presence of insulin for the action of growth hormone on cardiac glycogen. The administration of growth hormone to well-fed mice (at 9:00 A.M.) yielded increases of two-fold in the stores of cardiac glycogen (Fig. 3), but a total of four trials, using several experimental conditions, failed to yield comparable increases if the mice were injected at 4:30 P.M. (Table 3). The increment found in the mice injected in the morning could be duplicated if the animals were pretreated with insulin (0.1 U./kg.), or with a dose of glucose and forced alimentation (Table 3, C-3 and D-2). It is tempting to speculate that the peripheral activity of insulin is high at 9:00 A.M., following the large food intake during the night, and that the peripheral supply of insulin is low at 4:30 P.M. because mice feed only sporadically during the day, or that a reduced output of insulin during the day is the result of rhythmical controls. It is of interest here also that cardiac glycogen did not increase in the fasting mouse until the stores of liver glycogen began to increase (Fig. 1).

The fact that a dose of glucose superimposed upon the forced alimentation (Table 3, C-3) was more effective than forced alimentation alone may be explained in several ways. It is possible, for example, that the single dose of glucose supplied a greater stimulus to the pancreas than a slow absorption of glucose from the intestine. This requirement of insulin for the action of growth hormone receives some support from the work of Russell and Bloom (9), who found larger increments in cardiac glycogen if glucose was given in addition to growth hormone to a fasting rat. It might be argued that since growth hormone is as effective in an alloxan diabetic rat as in a normal rat, that insulin is not required (8). However, the alloxan diabetic rat must have some stores of insulin because a nearly total pancreatectomy of a rat will produce death within 48 hours unless insulin is supplied (15).

Acknowledgment

The author is deeply grateful to Dr. Jane A. Russell and to Dr. Alfred E. Wilhelmi for their generous gifts of purified growth hormone, for their criticism, and for their stimulating discussions during the course of this work.

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THE LOCALIZATION OF I^{131}-LABELED TRIIODOTHYRONINE IN THE PITUITARY AND BRAIN OF NORMAL AND THYROIDECTOMIZED MALE RATS

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ABSTRACT

The central nervous system and pituitary lobes of normal and hypothyroid male rats were both shown to be capable of concentrating triiodothyronine following intravenous injection of I^{131}-labeled triiodothyronine. The pituitary site of maximal localization was similar to that observed in the guinea pig, being greatest in the anterior lobe.

An autoradiographic study of the central nervous system and pituitary lobes showed a localization pattern similar to that seen in the guinea pig, being primarily associated with nuclear or cellular areas. However, the pronounced localization seen in the regions occupied by the neurosecretory structures in the guinea pig and rabbit was not observed in the rat. In addition, it was noted that the hippocampus, an area not actually studied in the other species, was practically autoradiographically negative in the rat.

Thyroidectomy decreased the rate of uptake of labeled hormone in the central nervous system. However, the peak concentration was increased in the central nervous system as well as in the adenohypophysis. The delay in attaining a peak concentration and the increased amount taken up were attributed to a deficiency in deiodinating mechanisms. A possible role for the thyroid hormone in maintaining cellular enzyme systems is suggested.

A possible relationship between the uptake curves of labelled triiodothyronine and the conjugated form of triiodothyronine is discussed.

Injections with radio iodinated serum albumen showed that the vascular space of the rat brain is very small, similar to the rabbit, and somewhat enlarged by hypothyroidism.

THE selective accumulation of I^{131}-labeled thyroxine and triiodothyronine by the pituitary and central nervous system (CNS) of the rabbit and guinea pig has been shown by several investigators (1, 2, and 3). In both species, the uptake of triiodothyronine was greater than the uptake of thyroxine under similar conditions, and it was observed that the pituitary lobe which concentrated the most triiodothyronine differed between species, the anterior lobe being most active in the guinea pig and least active in the rabbit. In investigations (3) on rats injected with labeled

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thyroxine, no observable difference between the two lobes in their affinity for the labeled hormone was observed, and they concentrated even less than did the median eminence. It was also observed that thyroidectomy had little or no effect on the organ/plasma ratios. Inasmuch as these experiments were done with thyroxine which is relatively poorly concentrated by both the brain and pituitary and since only one time interval after injection was used, it is difficult to assess what, if any, effect the thyroidectomy actually had, particularly since it would appear that the normal animals failed to concentrate thyroxine.

In view of these negative experiments with thyroxine in the rat and in consideration of the greater affinity of neural and pituitary tissues for triiodothyronine, the following experiments were performed with the objective of ascertaining, first of all: if the rat, like the rabbit and guinea pig, can localize and concentrate triiodothyronine within the pituitary and CNS; 2) whether the pituitary localizes the labeled hormone in a manner similar to the rabbit or to the guinea pig; and 3) what would be the effect of hypothyroidism on the uptake of thyroid hormone by the neural and pituitary tissues.

MATERIALS AND METHODS

The I^{131}-labeled triiodothyronine used in these experiments was obtained from the Oak Ridge laboratory of the Abbott Corporation, in a 50% propylene glycol solution. The material was checked for purity chromatographically and found to have a mean purity of 80% (72–87%). The method of dilution and chromatographic analysis has been previously described (1).

Male rats of the Wistar strain weighing from 295 gm. to 380 gm. were used in this investigation. There were 33 normal and 31 surgically thyroidectomized animals. The completeness of the thyroidectomy was checked by counting the amount of free iodide (131) taken up in the region of the thyroidal trachea after the animals injected with the labelled triiodothyronine were killed. If there was an appreciable uptake, indicating the presence of thyroidal tissue, the animal was discarded.

A third group consisted of 20 sham-operated animals. Inasmuch as the data obtained from this group was the same as that from normal animals, it will not be included.

The normal, thyroidectomized and sham-operated animals all received a dose of 0.5 μg. of triiodothyronine/kilogram (approximately 34 μc./kg.), intravenously into the jugular vein.

In addition to the above groups, a few animals were injected with a dose of 1 mc./kg. (10.5–27.5 μg./kg.). Four animals were used for an autoradiographic study, while 5 normal and 2 thyroidectomized animals provided fresh tissues for chromatographic analysis. Finally, 3 normal and 3 thyroidectomized animals were injected with 42 μc./kg. of Radio-Iodinated Serum Albumen (RISA) to obtain information relative to the vascular space in the various parts of the CNS studied.

All animals were maintained on a standard laboratory chow and water, *ad libitum*. The thyroidectomized animals were also given sufficient calcium lactate in their water to provide for a 1% solution. The thyroidectomy was performed from four to six weeks prior to the time of injection. The time intervals at which the animals were killed after injection were ½, 1, 2, 4 and 7 hours. The injection, counting, chromatographic and autoradiographic procedures were the same as those previously employed (1, 4).

Since one purpose of the investigation was to observe the effect of hypothyroidism on the ability of the pituitary and CNS to concentrate the labeled triiodothyronine, a statistical evaluation of the data was made by the method of Student "t" (5). However, it should be pointed out that there is an error of about 5% which occurs when counting low levels of radioactivity with the scintillation counters used to obtain the data. The addition of this error to the normal biological variation may mask or accentuate statistical differences. In view of this consideration, perhaps more attention should be directed to the shape and direction of the curves obtained than to the actual statistical differences between isolated points on the curves.

RESULTS

In the normal rat, the rate of clearance of I^{131} from the plasma after intravenous injection with I^{131}-labeled triiodothyronine was very rapid during the first ½ hour and much slower during the next six and one-half hours (Fig. 1). Thyroidectomized animals showed a similar rapid clearing of I^{131} from the plasma during the first ½ hour, but the level of radioactivity in the blood after this period remained at a slightly higher level than in the

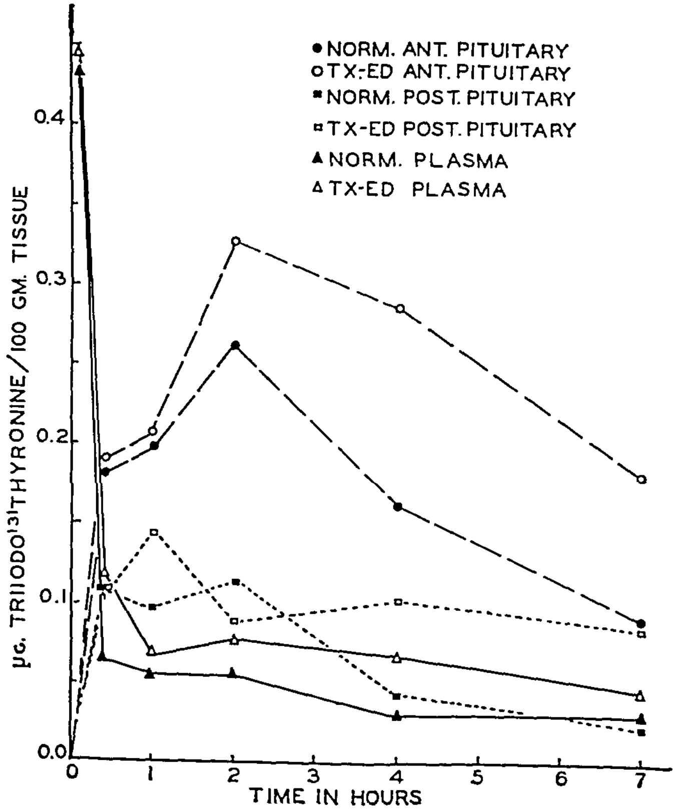

FIG. 1. The concentration of I^{131} in the pituitary and plasma at various time intervals after the intravenous injection of 0.5 μg./kg. of triiodothyronine. The solid symbols represent values from normal animals while the hollow symbols represent the values obtained from thyroidectomized animals.

normals. This difference was approximately 0.035 μg. of triiodothyronine /100 gm. of plasma for the last three hours. This difference was significant 4 hours after the time of injection ($P < 0.01$).

As the concentration of radioactivity in the plasma fell, there was a rapid uptake in both pituitary lobes, which was highest for the anterior lobe. The *greatest concentration was attained* 2 *hours after injection*, following which there was a slow decrease in the radioactivity present in both lobes. After thyroidectomy, the rate of uptake in the anterior pituitary was about the same during the first hour, with a slight increase in rate of uptake during the second hour. At the time of greatest triiodothyronine concentration there was 0.063 μg./100 gm. of tissue more in the thyroidectomized animals. The subsequent rate of fall in concentration closely paralleled that of the normal animals, but was maintained at a higher level. This difference was significant 4 hours after the injection of the hormone ($P < 0.01$). Note that in both the normal and thyroidectomized groups, there was a slight drop in the rate of uptake between the ½- and 1-hour period.

The posterior pituitary lobes in both the normal and thyroidectomized groups reached their maximal concentration at lower levels than did the anterior lobes, the time of greatest concentration being between the first and second hour. At no time during the course of the experiment was there an appreciable difference between the two groups.

In the CNS of normal animals, the rate of uptake of I^{131} after the injection of I^{131}-labelled triiodothyronine was relatively fast during the first ½ hour, during a period in which the plasma values were falling (Figs. 2 and 3). This was followed by a definite lag in the rate of uptake for the second ½ hour, after which there was again a rapid increase in the concentration of radioactivity within the tissues, with maximal concentration occurring at the end of 2 hours. The CNS concentration then fell slowly for the following 2 hours after which the concentration remained relatively constant, paralleling the concentration within the plasma.

In the thyroidectomized animals, the rate of uptake in the CNS during the initial 2 hours was for all intents and purposes identical to that of the normal animals (Figs. 2 and 3), with the exception of the isolated mammillary body in which no lag in rate of uptake was observed during the second ½-hour period. However, the highest concentration was not reached until 4 hours after injection in these animals. This concentration in the most responsive tissue (mammillary body) averaged 0.009 μg. of triiodothyronine/100 gm. tissue higher than that observed in the normal mammillary body (range 0.004–0.014), and the normal neuronal cellular area from which it differed most was the cerebral gray. The difference in the concentration of radioactivity within isolated mammillary body of the normal and thyroidectomized animals was significant one hour after the injection ($P < 0.05$). During the last 3 hours of the experiment, the con-

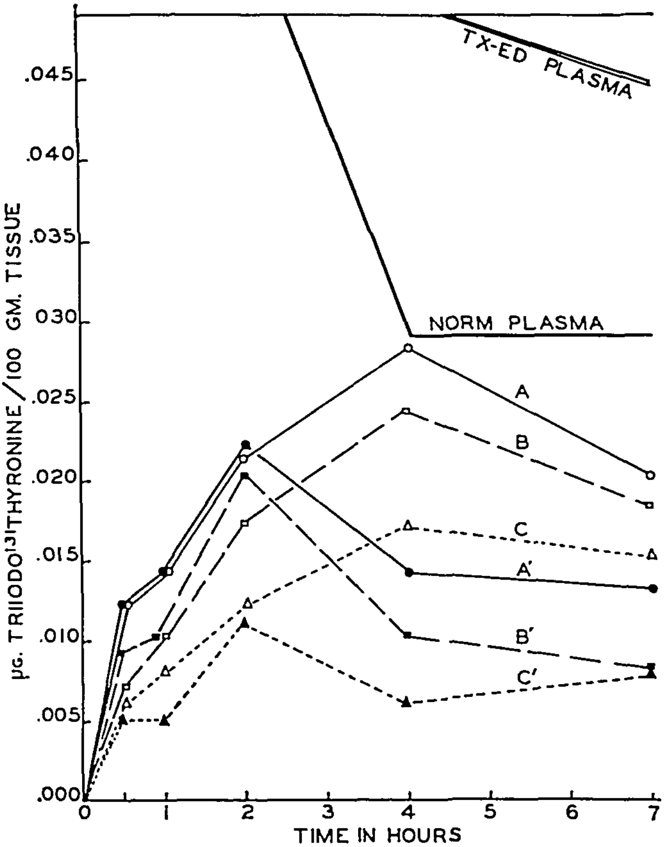

FIG. 2. The concentration of I^{131} in the cerebellum, cerebral gray and cerebral white matter at various time intervals after the intravenous injection of 0.5 μg./kg. of triiodothyronine. The solid symbols represent values from normal animals while the hollow symbols represent values from thyroidectomized animals. The circles indicate the cerebellum (A,A^1), the squares the cerebral gray (B,B^1), and the triangles the cerebral white matter (C,C^1).

centration of radioactivity in the neural tissues of thyroidectomized animals fell at a rate comparable to the rate of fall in the plasma level of radioactivity. This rate appeared to be somewhat faster than that observed in the normal animals, where the rate of fall, while less, also paralleled the concentration maintained in the plasma.

It is to be noted in both normal and thyroidectomized animals, that the concentration of radioactivity was greater in the cerebral gray matter than in the white matter, being significantly higher ($P < 0.05$) in the normal group 1 and 4 hours after the injection.

Chromatographic separation of normal plasma samples showed that as the concentration of radioactivity in the plasma drops sharply with time (Fig. 1), the level of radioactivity attributable to triiodothyronine also dropped, with the greatest rate of fall occurring in the first one hour (Fig.

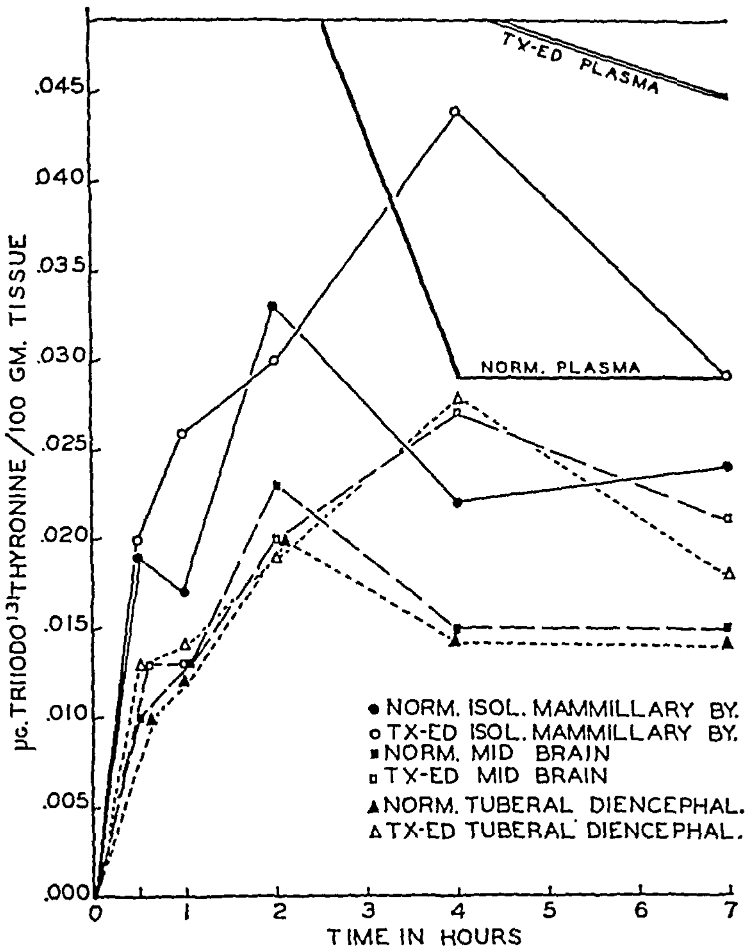

FIG. 3. The concentration of I^{131} in the isolated (isol.) mammillary body (by.), mid brain and tuberal diencephalon (diencephal.) at various time intervals after the intravenous injection of 0.5 μg./kg. of triiodothyronine. The solid symbols represent values from normal animals, while the hollow symbols represent values from thyroidectomized animals.

4). This drop in plasma triiodothyronine was accompanied by an increasing per cent of the total plasma radioactivity which could be attributed to free iodide. A somewhat different picture was presented by the thyroidectomized group, where the per cent of total plasma radioactivity attributable to triiodothyronine fell off much less and was accompanied by a much smaller increase in plasma iodide levels.

The more rapid rate of deiodination of triiodothyronine observed in the plasma of normal animals was also seen in the adenohypophysis and in the CNS (Table 1). These differences in the percents of the various forms in which the radioactivity was present are considered to indicate some difference in the abilities of the adenohypophysis and CNS of the two groups to handle the injected material. With the exception of the posterior pituitary, the tissues in the normal group had less triiodothyronine and more iodide than did comparable tissues in the thyroidectomized group. There

TABLE 1. THE METABOLITES OF TRIIODO^{131} THYRONINE PRESENT IN THE PITUITARY, BRAIN AND PLASMA OF THE NORMAL AND HYPOTHYROID MALE RAT AFTER INTRAVENOUS INJECTION OF 1.0 MC./KG.* OF THE LABELED HORMONE

Tissue	Triiodothyronine		Iodide		Residue	
	N	Tx	N	Tx	N	Tx
1 Min Plas	78.0†	76.0†	4.0	4.1	7.3	17.2
2 Hour Plas	42.7	57.1	26.0	15.5	28.9	25.0
Ant. P.t.	57.5	65.7	7.6	6.0	29.4	25.3
Post P.t.	55.4	39.7	2.9	11.0	33.0	37.2
Iso. Mam. Body	55.0	73.2	10.7	5.4	26.8	18.6
Iso. Tub. Cin.	34.1	58.3	23.9	10.4	35.3	24.8
Cerebral Grey	30.6	69.0	11.8	4.2	41.7	23.4
Cerebral White	38.2	59.9	12.1	9.8	35.2	27.2
Cerebellum	61.1	65.5	4.6	6.4	29.0	24.3

N = Normal, Tx = Thyroidectomized.

* Dose of Triiodothyronine = 25.0 μg./kg. for normal rat and 21.6 μg./kg. for the thyroidectomized rat.

† Sum of 6 animals receiving 0.5 μg./kg.

was also a greater I^{131} per cent listed as Residue (unidentified iodine-containing substances) found in the tissues of normal animals.

Since the plasma of the thyroidectomized group maintained a higher level of total radioactivity than that of the normals, the increased radio-

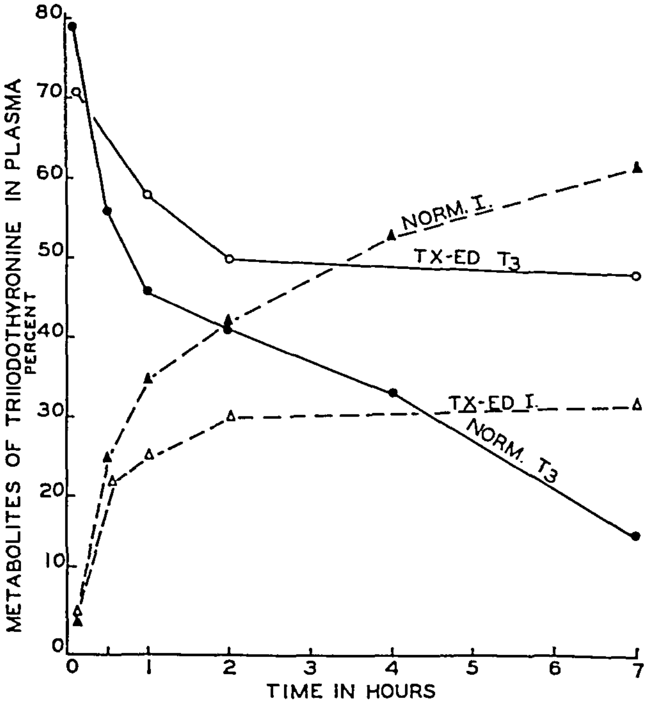

FIG. 4. A graphic representation of a chromatographic analysis of the distribution of the metabolites of I^{131}-labeled triiodothyronine in the plasma of normal and thyroidectomized animals after the intravenous injection of 1 mc. of the labeled hormone/kg. (Norm. = normal, Tx-ed = thyroidectomized, I = iodide, and T_3 = triiodothyronine).

activity in the tissues of the thyroidectomized animals might be due to the increased radioactivity in the trapped blood. If true, the time of highest concentration in the CNS and pituitary of the thyroidectomized animals would be expected to occur at the same time as in the normals. Even though the times at which the maximal concentration occurred differed, it was possible that a considerable portion of the increased activity in tissues from thyroidecomized animals might be due to the radioactivity of the trapped blood.

The experiment with RISA injected animals (Table 2) showed that the radioactivity in the trapped tissue plasma was not great enough to account for the increased radioactivity in the thyroidectomized group, despite an increase in the CNS blood volume which occurred in most of the CNS in the thyroidectomized animals (Table 2): (i.e., calculation of the increase in the vascular space of the isolated mammillary body, over that normally found, showed it to be 0.0113%. When this is multiplied by the difference in blood level of radioactivity at the time of greatest divergence (0.035 μg. at 4 hours), one observes that the 0.00040 μg. of triiodothyronine/100 gm. of mammillary body which would have to be added to the normal or subtracted from the thyroidectomized animals to correct for differences in blood volume or total plasma radioactivity, would in no way change the result).

Table 2. A comparison of the concentration of I^{131}-labeled triiodothyronine (0.5 μg./kg.) (−34.0 μc./kg.) and radio iodinated serum albumen (RISA) (42 μc./kg.) in the CNS and pituitary of normal and thyroidectomized rats as reflected by the organ/plasma ratio

	Triiodothyronine One hour after injection		RISA One and one-quarter hours after injection (=plasma space)		
	Normal	Thyroid-ectomized	Normal	Thyroid-ectomized	Change after Thyroidectomy
Ant. Pituitary	3.45	2.87	0.089	0.086	−0.003
Post. Pituitary	1.89	1.88	0.093	0.048	−0.045
Isol. Mammillary Body	0.311	0.278	0.0057	0.0170	+0.0113
Isol. Tuber Cinereum	0.284	0.264	0.0077	0.0110	+0.0033
Tuberal Diencephalon	0.179	0.192	0.0052	0.0050	−0.0002
Chiasmic Diencephalon	0.181	0.171	0.0056	0.0063	+0.0007
Cerebellum	0.249	0.202	0.0078	0.0150	+0.0072
Cerebrum	0.150	0.146	0.0054	0.0090	+0.0036
Cerebral Gray	0.168	0.157	0.0075	0.0126	+0.0051
Cerebral White	0.088	0.113	0.0049	0.0063	+0.0014

While the plasma volume changes in the CNS of thyroidectomized animals were in all but one instance positive, the changes in the pituitary were negative, and it is interesting to note that the one part of the CNS which showed a slight decrease in plasma volume is associated with the posterior pituitary.

The autoradiographic localization of what was shown to be at least partly triiodothyronine (Table 1) and its metabolic breakdown products was within those parts of the CNS most rich in cell bodies, being much more concentrated in the gray than in the white matter. While the pattern of localization was similar to that seen in rabbits and guinea pigs, it was somewhat more diffusely distributed. Other relatively clear nuclear localizations were noted throughout the neuraxis (Fig. 5), with a particularly marked localization in the arcuate nucleus in the median eminence. It is interesting to note that the hippocampus, which possesses a very dense layer of pyramidal cells, was much less reactive than the cerebral cortex. No pronounced accumulations of radioactivity could be associated with the neurosecretory areas, as were shown in the rabbit and guinea pig (1, 2).

DISCUSSION

This investigation was designed to consider three questions, which were: a) will the CNS and pituitary tissues of the rat concentrate triiodothyronine? b) which pituitary lobe will be the most active in concentrating the labeled hormone? c) what will be the effect of hypothyroidism on the ability of these tissues to concentrate the labelled hormone?

There is little doubt but that the CNS and pituitary tissues in the rat, like those of the rabbit and guinea pig, are capable of concentrating L-3:5:3′-triiodothyronine. Furthermore, the labeled hormone was cleared from the plasma at a rate equal to that seen in the rabbit or guinea pig (1, 2). While the organ/plasma ratios never exceeded one in either normal or thyroidectomized animals (Table 2), they still exceeded the plasma and hence the water space of the brain (Table 2); so it must be concluded that the material had at least passed through the capillary walls. Also, since electronmicroscopy of the rat brain (6, 7) reveals practically no extracellular space, it is probably safe to conclude that the radioactive material is in either the neuronal or glial elements. The nature of the material localized in the CNS, as in the guinea pig and rabbit, appeared to be partly triiodothyronine and partly its metabolic breakdown products (Table 1). Inasmuch as free iodide is not taken up by the brain within the time limits of these experiments (2) and apparently must be incorporated into the thyroid hormone before being taken up by neural tissues (8), the iodide observed in the CNS must be derived through intracellular deiodination processes.

The autoradiographic localizations are similar to those observed in the guinea pig and rabbit, although a specific localization in the neurosecretory area was not observed. This may, in part, be related to the fact that there is very little histologically visible neurosecretory product in the rat (9) as compared with either the rabbit (10) or guinea pig (unpublished observation).

The observation that the hippocampus with its densely packed layer of pyramidal cells was autoradiographically negative was surprising. This was

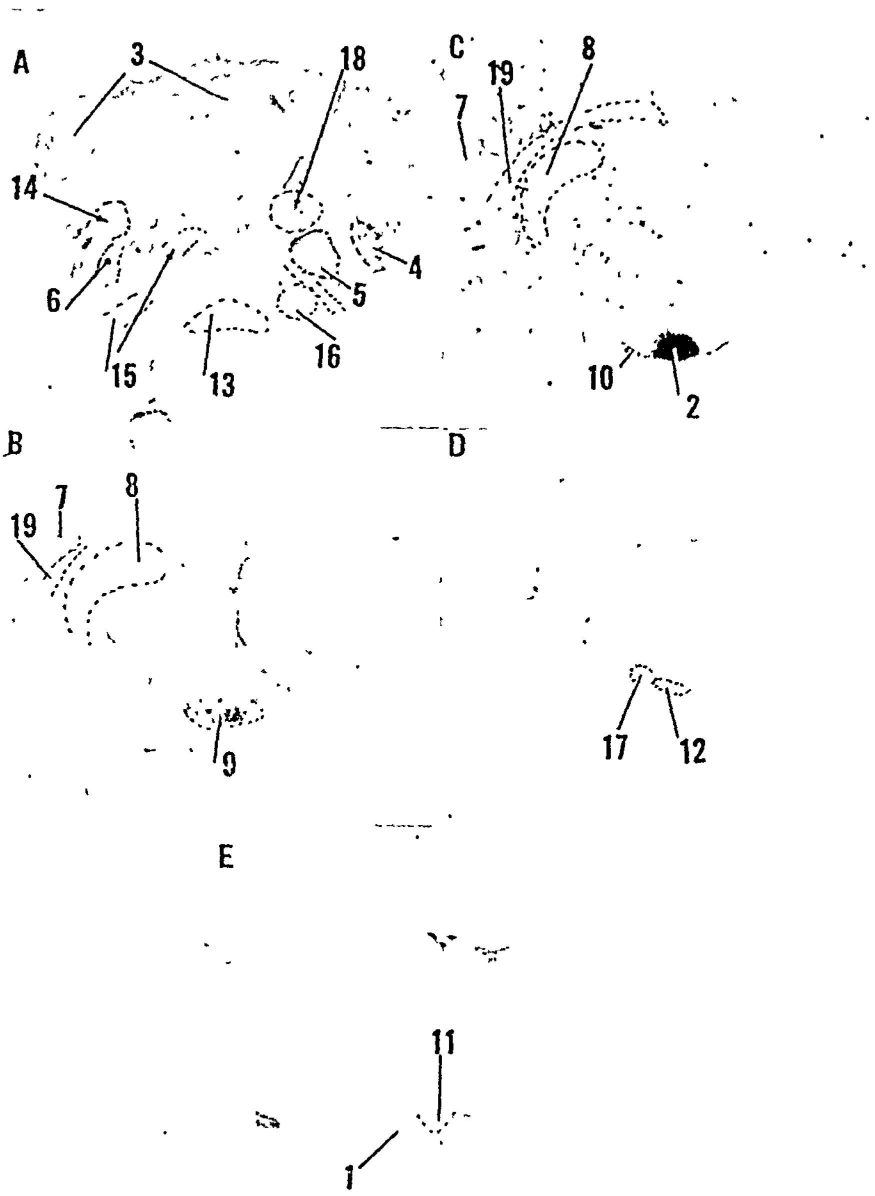

FIG. 5. An autoradiographic representation of the sites of localization of I^{131} in the rat brain after injection of I^{131}-labelled triiodothyronine. A. Coronal section through the cerebellum and pons; B. Coronal section through the caudal diencephalon and associated cerebral hemispheres; C. Coronal section through the median eminence and associated cerebral hemispheres; D. Coronal section through the rostral diencephalon and associated cerebral hemispheres; E. Coronal section through the pituitary. The numbers refer to the following: 1. Adenohypophysis, 2. Arcuate nucleus, 3. Cerebellum, 4. Cochlear nuclei, 5. Descending trigeminal nucleus, 6. Descending trigeminal tract, 7. Gray matter, 8. Hippocampus, 9. Mammillary body, 10. Median eminence, 11. Neurohypophysis, 12. Optic tract, 13. Pons (fibers and nuclei), 14. Restiform body, 15. Seventh nerve (fibers), 16. Seventh nerve (nucleus), 17. Supraoptic nucleus, 18. Vestibular nuclei, 19. White matter.

particularly true since S^{35}-labelled methionine (11) and S^{35}-labeled L-cystine (12) have shown a rather pronounced localization in this band of cells. The observations with the labeled cystine are of further interest in that the autoradiographs were made at various time intervals over a 24-hour period. They showed that the hippocampal pyramidal cell area concentrated the amino acid more rapidly than did the cerebral cortex. It was felt that this indicated differences in the rates of amino acid incorporation into the neural protein in these areas. The observation that triiodothyronine is not well localized in the hippocampus by autoradiographic procedures as compared with the cerebral cortex, also implies some difference between the two areas in the rate at which the normal metabolic processes may be proceeding.

TABLE 3. COMPARISON OF CENTRAL NERVOUS SYSTEM SITES OF DEIODINATING ACTIVITY WITH THE CONCENTRATIONS OF I^{131}-LABELED TRIIODOTHYRONINE IN THE SAME AREAS

Tissue	Deiodinating* activity	µg. Triiodothyronine/100 gm. CNS tissue at different time intervals (in hours)					
		½	1	2	4	7	
Whole Brain	100	—	—	—	—	—†	Tissues high in Deiodinose Activity
Cerebrum	172	.008	.009	.013	.009	.008	
Cerebral Cortex	240	.010	.010	.020	.010	.008	
Cerebellum	82	.012	.014	.022	.014	.013	Tissues low in Deiodinase Activity, but relatively high in concentration of Triiodothyronine
Medulla	37	.012	.013	.023	.013	.014	
Mid Brain	13	.012	.013	.023	.015	.015	
Isolated Mamillary‡ Body	—	.021	.017	.033	.022	.024	

* From Tata (13).
† Not determined in this study.
‡ Not determined in TATA's study.

In previous papers (1, 2) an attempt was made to correlate the higher localizations of thyroid hormone in the hypothalamus with some specific role which it might play in this area. While this still may be true, there also exists the possibility that the amount localized may depend on the amount of deiodinase present, being low in areas rich in deiodinase. The possibility of such a relationship was suggested by comparing the data on triiodothyronine concentration with Tata's data on deiodinating activity in the brain (13, Table 3). It was noted that areas relatively rich in deiodinating activity, like the cerebral cortex, had a lower concentration of triiodothyronine than did the areas low in deiodinating activity, like the mid brain, where the triiodothyronine concentrations were higher. One would suspect on this basis that the isolated mammillary body might have an even lower concentration of a deiodinating enzyme if this analogy is true. Unfortunately, no information is available for this area. If this relationship is true, the hippocampus might be expected to contain a very high level of deiodinase activity, inasmuch as the autoradiographic concentration of triiodothyronine appears to be so low in this structure. If the relative concentrations of triiodothyronine in the brain and pituitary do depend primarily on the relative concentrations of deiodinase, one would have to presume that the adenohypohysis, the arcuate nuclei and the neurosecretory region in the rabbit and guinea pig would be practically free of deiodinase. Inasmuch as chromatographic studies have shown that deiodination is occurring in these areas, one must conclude that while the relative concentrations of triiodothyronine which were found may depend, in part, upon the deiodinating activity within that region, this can not be the only factor involved.

The second question pertained to the pituitary site of maximal localization, and it was observed that the anterior lobe, as in the guinea pig, concentrated considerably more of the labeled hormone than did the posterior lobe. Why the rat and the guinea pig differ from the rabbit in this respect is still unknown.

That the thyroid hormone plays some role in the normal function of the brain has long been accepted. However, the nature of this role has been most elusive. There have been many investigations on the utilization of oxygen and glucose (14–17), studies on growth and maturation and vascular supply (18–22), and effects on the EEG pattern and nerve excitability (23–25), yet, the precise manner whereby the thyroid hormone affects normal brain function has not yet been clearly ascertained. Timiras and Woodbury (26, 27), have reported a most interesting series of results which appear rather clearly to implicate the thyroid hormone in aiding the adrenocorticoids in the control of the electrolyte levels within the nerve cells. Thus, the third question dealing with the effects of thyroidectomy relates to the overall question of the role of the thyroid hormone in CNS function.

The two most discernible effects of thyroidectomy were that there was a slight increase in the vascular space in the thyroidectomized animals (Table 2) and that while the tissues of the thyroidectomized animals concentrated more of the labeled hormone (Figs. 1, 2, 3, 4), it has taken them longer to do so. The adenohypophysis is the only real exception in that it achieves its maximal concentration at the same time in both groups. The change in vascular volume induced by hypothyroidism is not surprising since Eayrs (21) has previously shown that hypothyroidism affects the vascular system.

A third observation, based on chromatographic analysis of the tissues and plasma reveals a decrease in deiodination in the thyroidectomized animals (Fig. 4, Table 1). This evidence for a disruption in an enzyme function suggests that one role for the thyroid hormone may be in the maintenance of intracellular enzyme systems. While this is probably not a role restricted to neural tissues, or to deiodinating enzymes alone, nothing further can be said at this time.

While the increase in the concentration of radioactivity in the tissues of thyroidectomized animals may, in part, be due to the higher concentration in the plasma and the slightly increased vascular space in CNS tissues (Table 2), the total increase attributable to these factors was not sufficient to account for the change in concentrations. Also, if these were the only factors involved, one would have suspected that the time of maximal concentration would have coincided with that of the normal animals, as it does in the pituitary. Inasmuch as the space alterations in the pituitary were negative (Table 2) and since the increase in plasma radioactivity was not enough to account for a rise in anterior pituitary radioactivity, some other factor must also be concerned with the increase observed in the adenohypophysis.

Another explanation for an increased concentration of radioactivity in the CNS (3) might be that there is no endogenous thyroid hormone to dilute the intracellular concentration of the labeled hormone, thus increasing the potential thyroid hormone space. If this was the only answer one would suspect that the time of maximal concentration would be the same as that observed for normals. While this factor may be quite important in the pituitary gland where the time of maximal uptake is the same for both groups, it does not seem to be applicable to the CNS where there is a delay in reaching the greatest concentration.

A further possibility for the increased time necessary to reach a maximal concentration in the CNS is that there may be a reduced rate of utilization of the incorporated hormone by the thyroidectomized animal. This may be associated with a decrease in the rate of deiodination in these tissues (Table 1). Such a decrease in the deiodination of the triiodothyronine might tend to permit a higher accumulation of the hormone within the tissue before the rate of deiodination exceeds the rate at which the labelled hormone is concentrated. Once this point is reached the deiodination appears to progress at a slower rate in the thyroidectomized animals (Fig. 4), and levels off after the fourth hour. In the normals the per cent of free iodide in the plasma continued to increase with time.

In the presentation of the data, it was noted in almost every part of the CNS and in the pituitary that there was a short lag in the rate of uptake which occurred in the second half-hour period. From the manner in which the uptake curves progress, it would appear that all organs are participating in an initial rapid clearing of triiodothyronine from the blood during the first $\frac{1}{2}$ hour (Figs. 1, 2, 3). This has also been noted for the adrenals (unpublished data) and liver (28). There then follows a decreased rate of concentration in the tissues. The time when the rate of concentration of radioactivity by the CNS again shows an increasing slope (1 hour after the injection) corresponds well with the time when the glucuronide-triiodothyronine complex formed in the liver starts to appear in the plasma in sizeable concentrations (15). The pathway for entry of this complex into the blood appears to be in part through biliary excretion since the bile is very rich in this complex, followed by intestinal resorption (4). This complex is then slowly cleared from the plasma, being practically gone within two hours, when the levels of concentration in the tissues again start to fall. During the next two hours, this complex again starts to appear slowly in the plasma, becoming relatively prominent four hours from the beginning of the experiment. This corresponds to the time when the kidney has been shown to contain large amounts of the complex (29) and coincides with a period in which the rate of fall of CNS radioactivity levels off, as does the fall in plasma activity, possibly due to reentry into the vascular channels of a fresh supply of the conjugated form of triiodothyronine from the kidney. The fact that the levels of concentration do not rise is probably due to the very low levels of hormone with which one is dealing at this time. Since not even traces of the glucuronide complex are shown in the pituitary lobes, muscle (15) or CNS by chromatographic studies, it is concluded that it is only a transport form of the thyroid hormone which is rapidly dissassociated to give up the free triiodothyronine which enters the tissue. From this discussion, it would appear that the CNS concentration of triiodothyronine is very readily affected by blood levels of either the free or conjugated form of the hormone.

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THE DESTRUCTION OF GLUCAGON BY THE BLOOD PLASMA FROM VARIOUS SPECIES[1]

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ABSTRACT

Glucagon is hydrolyzed *in vitro* by blood plasma from various species. The highest rates occur with the plasmas from monkey and rat. Much lower rates of glucagonolysis are produced by plasmas from guinea-pig, chicken, man, rabbit, cow, dog and cat. The addition of streptokinase to the incubation mixture increases markedly the glucagonolytic activity of the plasmas from man, monkey and cat. In the presence of trace amounts of human plasma or of a crude euglobulin fraction of human plasma, streptokinase increases the glucagonolytic activity of the plasmas (or euglobulin fractions of such plasmas) from the various species. The data indicate that plasmin is responsible for the glucagonolytic action of plasma and that the activation of plasminogen by streptokinase is dependent upon a 'proactivator' which is present in human, monkey and cat plasmas, and absent or very low in the plasmas from the other species. It is proposed that the glucagonolytic action of native plasma is due to the release of endogenous activators of plasminogen and reflects a more general action of plasmin.

HUMAN plasma contains an enzyme system which catalyzes the destruction of adrenocorticotropin (1, 2, 3), somatotropin (3) and glucagon (3). In the presence of streptokinase, this hydrolytic activity of human plasma is increased markedly (3). The effect of streptokinase suggested that the active proteolytic enzyme, plasmin, formed from the conversion of the inactive precursor, plasminogen, is responsible for the destruction of these hormones. In accord is the marked acceleration of the destruction of the three hormones by a crude euglobulin fraction (plasminogen) of human plasma which occurs in the presence of streptokinase (3), the rapid rate of destruction produced by purified preparations of human plasmin (3) and the splitting of corticotropin A by bovine plasmin (4).

The blood plasma or serum from many infra-human species does not respond to amounts of streptokinase which readily produce an increase in the proteolytic or fibrinolytic activity of human plasma (5, 6, 7, 8, 9, 10,

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11, 12, 13, 14). In the presence of even minute amounts of euglobulin from human plasma, however, streptokinase catalyzes the conversion of rabbit, guinea-pig, dog, avian, and bovine plasminogen to plasmin (8, 9, 10, 13, 14, 15). Consequently, it has been proposed that the catalysis of the conversion of plasminogen to plasmin by streptokinase is dependent upon a co-factor or 'proactivator' which is presumed to be present in relatively large concentrations of human plasma but not in that of the dog, rabbit, guinea-pig, chicken, duck and rat (8, 13, 14). Accordingly, if plasmin is responsible for the destruction of some of the peptide hormones, the presence of streptokinase in amounts which are effective with human plasma should have relatively little or no effect on the proteolytic activity of the plasma from the above sub-human species. In the presence of the human plasma 'proactivator,' however, streptokinase should readily increase the proteolytic activity of the plasma. To test this thypothesis, the degradation of glucagon *in vitro* by fresh plasma from man, monkey, cow, dog, cat, rabbit, guinea-pig, rat and chicken was studied.

METHODS

Venous blood was drawn into heparinized syringes from men, monkeys, dogs, cats, chickens and cows. Cardiac blood was taken from rabbits, guinea-pigs and rats. In all instances the plasma was separated rapidly. The activity of the native and streptokinase-treated plasma was determined by incubating 1 ml. plasma with 0.5 ml. buffer containing 0.2 μmole of crystalline glucagon[2] (M.W. = 10,000) and 0.5 ml. MacIlvaine buffer (16) at pH 7.8 (without and with 1,000 units streptokinase). The streptokinase preparation (Varidase®)[3] assayed approximately 5,000 units per mg. The mixtures were incubated in test tubes in a Dubnoff shaker at 37° C for 90 minutes at which time the reaction was stopped by the addition of an equal volume of 10 per cent trichloracetic acid (TCA). Controls consisted of the same mixtures except that the plasma was added after the TCA. The glucagon comprising the substrate contained tracer quantities of I^{131} labeled glucagon prepared by the procedure described by Bauman *et al.* (17). The radioactivity of the whole TCA precipitated incubation mixture and of the TCA soluble portion were assayed with a well type scintillation counter and after appropriate corrections the amount of protein that was hydrolyzed was computed as described previously (18). An increase in the per cent of the total radioactivity which appears in the TCA filtrate indicates the hydrolysis of the substrate (3).

The effect of human 'proactivator' on the degradation of glucagon by streptokinase-treated plasma was determined by incubating 1 ml. plasma with 0.5 ml. buffer containing 0.2 μmole of crystalline glucagon and 0.5 ml. buffer at pH 7.8 containing 1,000 units streptokinase without and with 0.01 ml. human plasma. The human plasma was incubated with the streptokinase for 5 minutes before the addition of the other constituents of the incubation mixture. The action of the 0.01 ml. human plasma plus streptokinase was determined by incubating the same mixture except that the animal plasma was added after the precipitation with TCA.

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Crude euglobulin (plasminogen) fractions of human and animal plasmas were prepared by precipitating fresh plasma with 19 volumes of distilled water and adjusting to pH 5.2 with 0.5 per cent acetic acid (19). The precipitate from each ml. of plasma was resuspended in 1 ml. of MacIlvaine's buffer at pH 7.8. Each incubation mixture consisted of 1 ml. of the animal plasma euglobulin solution, 0.5 ml. buffer containing 1,000 units streptokinase without and with 0.01 ml. human plasma euglobulin solution and 0.5 ml. buffer containing 0.2 µmole glucagon. The human plasma fraction was incubated with the streptokinase for 5 minutes before the addition of the other constituents of the incubation mixture. In addition, the activity of the human euglobulin fraction was determined by incubating the same mixture except for the animal plasma fraction which was added after the precipitation with TCA.

Radioautographs of chromatograms of various incubation mixtures were prepared by the procedure described by Tong, Taurog and Chaikoff (20).

RESULTS

Native plasma (0.5 ml. per ml. incubation mixture) from all the species studied destroyed glucagon (0.1 µmole per ml. incubation mixture) to some degree during the 90 minute incubation period. The data (mean ±S.E.) obtained with plasma from the various species is illustrated in Figure 1. The highest rate of glucagon degradation was produced with monkey plasma (14.2 ±1.5%). Untreated rat plasma also produced a rela-

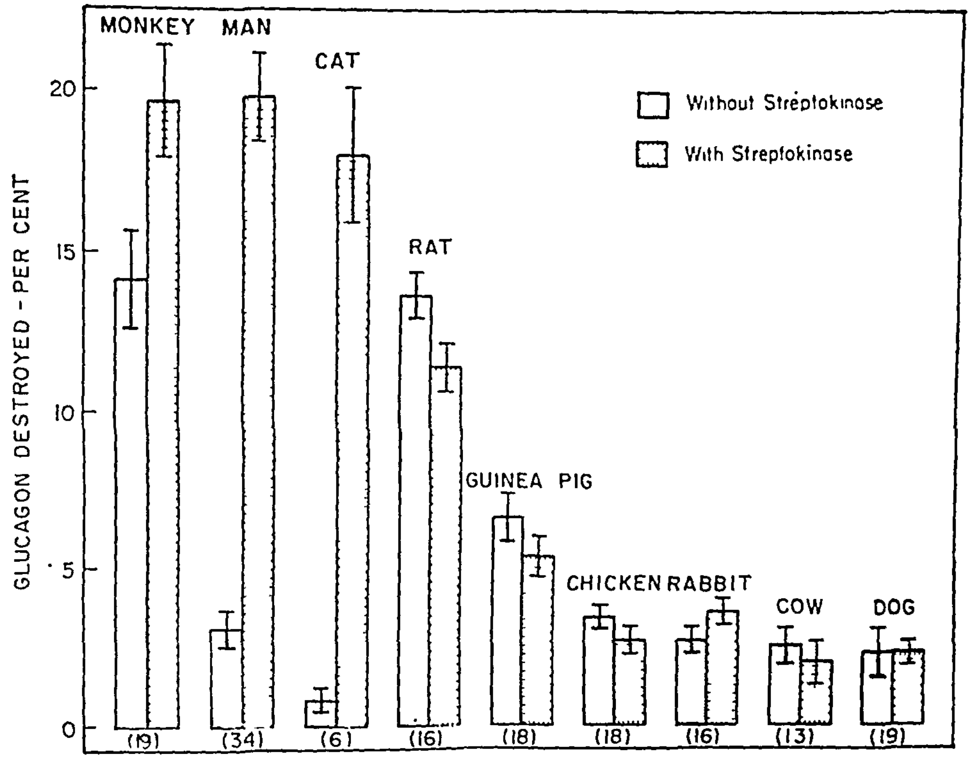

Fig. 1. Degradation of Glucagon by the Plasma from Various Species.

The per cent of glucagon (mean±S.E.) destroyed in 90 minutes incubation with plasma, without and with streptokinase, was computed as indicated in text. The numerals refer to the number of samples that were studied.

tively high rate of glucagon destruction (13.7 ±0.7%). The mean activity of 34 samples of human plasma (3.1 ±0.6%) was lower than that observed earlier (3) and in the range of the activities obtained with the native plasmas from guinea-pig, rabbit, chicken, dog and cat.

The addition of streptokinase (1,000 units per ml. plasma) to the incubation mixture resulted in a marked increase in the rate of glucagon degradation produced by human, monkey and cat plasmas (Fig. 1). No statistically significant change in the activity of the plasma from any of the other species occurred in the presence of streptokinase. Radioautographs of chromatograms of aliquots of the incubation mixtures containing glucagon, plasma and streptokinase are illustrated in Figure 2. 20 λ of each incubation mixture was taken at 0, 1 and 2 hours and applied as a streak to the pages. Although it was necessary to overload the chromatograms to do so, the radioautographs reveal that some degree of glucagonolytic activity is exerted by the plasma from each of the species.

The influence exerted by the 'proactivator' present in human plasma is illustrated in Figure 3 which depicts the data obtained (mean ±S.E.) after incubation of the plasmas (0.5 ml. per ml. incubation mixture) with glucagon (0.1 μmole per ml. incubation mixture) and streptokinase (500 units per ml. incubation mixture) with and without human plasma (0.005 ml. per ml. incubation mixture). Appropriate corrections were made for the activity of the human plasma plus streptokinase. Only those species whose plasmas were not activated by streptokinase were studied. It is evident that the presence of the 'proactivator' in the relatively small volume of human plasma produced a marked acceleration in the rate of glucagon degradation by the plasma from the guinea-pig and the dog. The activity of rat plasma was moderately increased in the presence of the 'proactivator' while the activity of rabbit plasma was not changed significantly. The activity of the streptokinase-treated plasmas from chicken and cow was inhibited by the human plasma.

In order to reduce the quantity of plasmin inhibitors present in the human and animal plasmas, the effect of a euglobulin fraction prepared from fresh human plasma (equivalent to 0.005 ml. plasma per ml. incubation mixture) on the activity of a similar animal plasma fraction (equivalent to 0.5 ml. plasma per ml. incubation mixture) was studied after a preliminary incubation of the former with streptokinase (500 units per ml. incubation mixture). Appropriate corrections for the activity of the human plasma factor plus streptokinase were made. The data (mean ±S.E.) obtained are illustrated in Figure 3. As with the native plasma, the streptokinase-treated human euglobulin fraction increased the activity of the euglobulin fraction of the guinea-pig plasma and, to a lesser degree, of the dog plasma fraction. In addition, the streptokinase-treated human plasma fraction produced a more marked enhancement in the proteolytic activity of the rat and rabbit euglobulin plasma fractions than did an equivalent amount

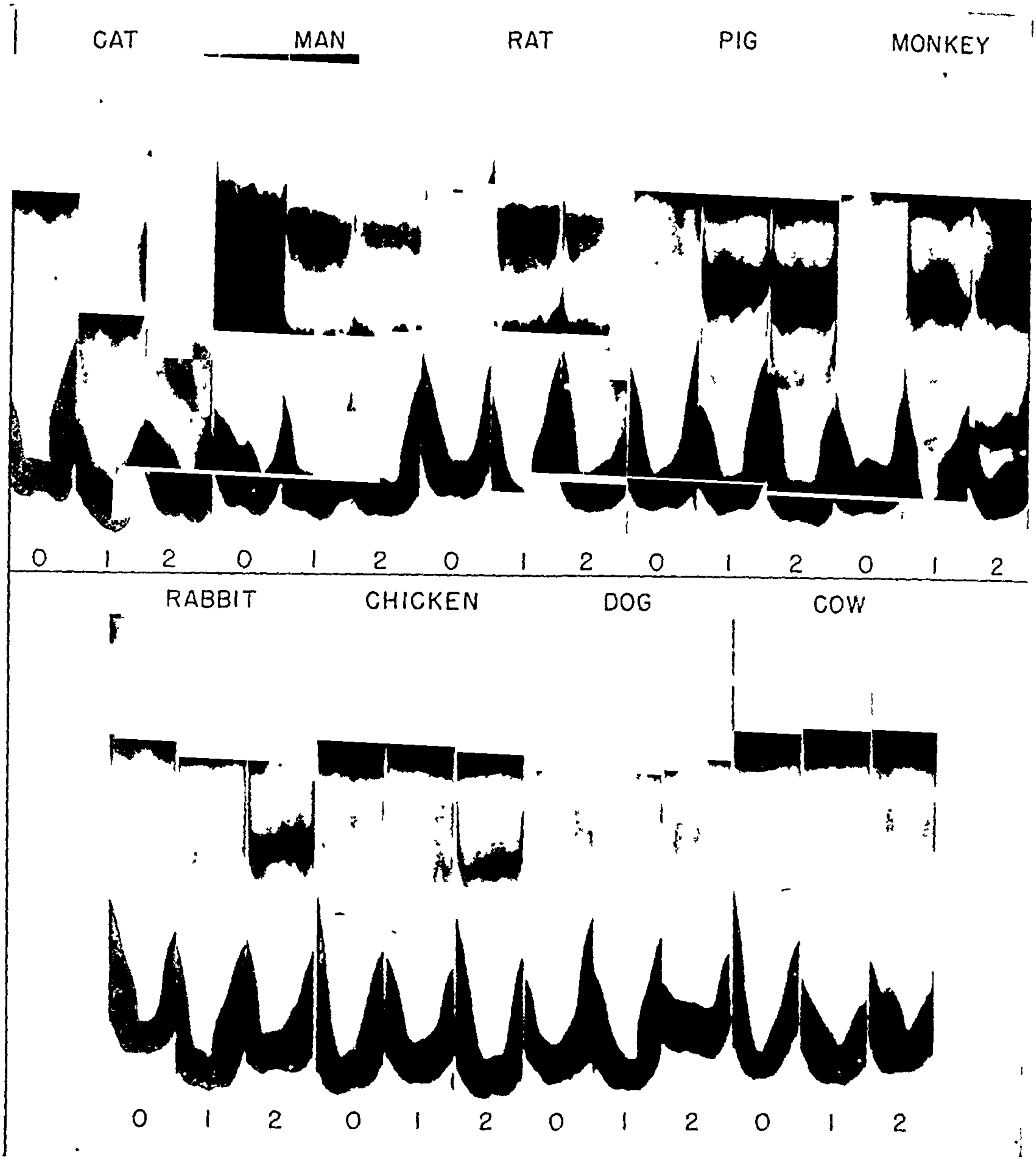

FIG. 2. Degradation of Glucagon by Streptokinase-Treated Plasma from Various Species.

Radioautographs of descending chromatograms of 20 λ aliquots of incubation mixtures removed at designated intervals (hours), streaked on paper and chromatographed as indicated in text.

of native human plasma. Further, whereas the native human plasma produced an inhibition in the activity of the plasma from chickens and cows, the euglobulin fraction of human plasma produced a marked increase in the activity of the bovine plasma fraction and a small but statistically significant increase in the activity of the chicken plasma fraction.

DISCUSSION

The data reported herein reveal that plasma from monkeys and rats can destroy glucagon at a relatively high rate while that from man, cat,

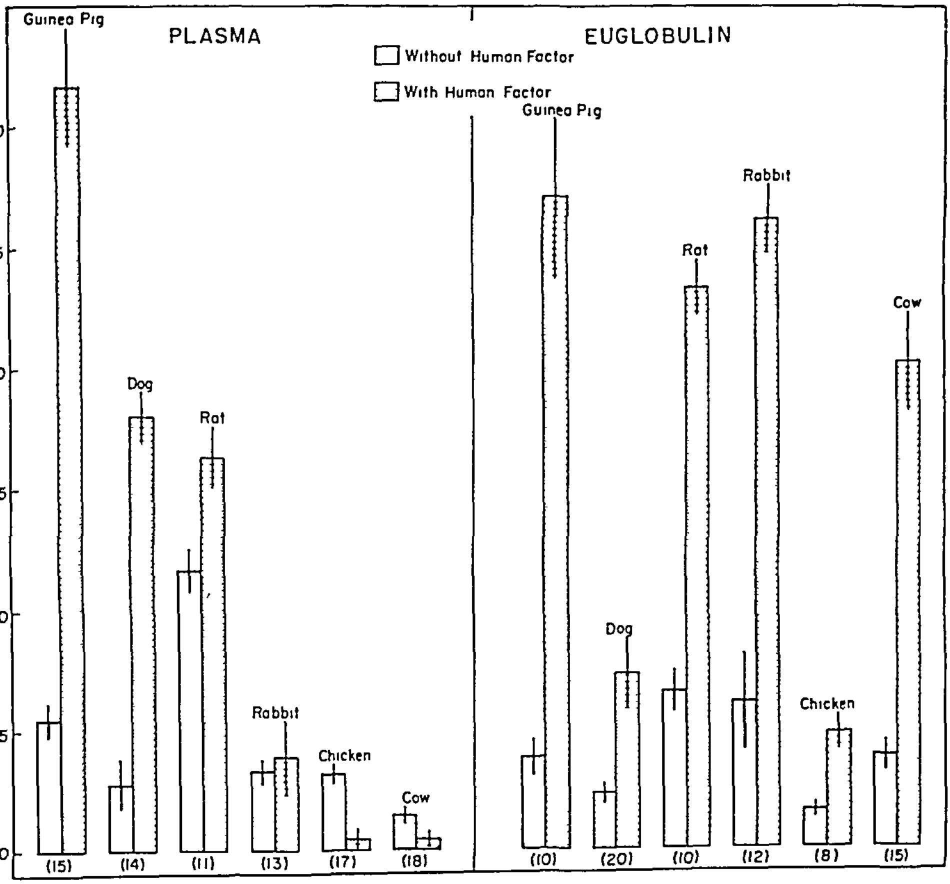

FIG. 3. The Effect of Human 'Proactivator' on the Glucagonolytic Action of Streptokinase-Treated Plasmas from Various Species.

The methods employed are indicated in text. The numerals refer to the number of plasmas studied.

guinea-pig, rabbit, chicken, dog and cow exerts a much lower rate of destruction. If it be assumed that 0.1 μg. glucagon is equivalent to one cat unit (the amount per kilogram of body weight which produces a rise of 30 mg. per cent in the blood sugar of the cat) (21), then the rates of destruction of glucagon by monkey and rat plasmas in 90 minutes are 56.8 ± 6.0 and 54.8 ± 2.8 cat units respectively. Even the much lower rates of hydrolysis produced by guinea-pig, chicken, human, rabbit, bovine, dog and cat plasmas are not negligible when expressed as cat units of glucagon hydrolyzed per ml. plasma per 90 minutes, viz., 25.4 ± 3.2, 13.6 ± 1.6, 12.4 ± 2.4, 10.8 ± 1.6, 10.0 ± 2.4, 9.2 ± 2.0, and 3.2 ± 1.6 units respectively.

That the hydrolysis of glucagon by the native plasma of man is due to plasmin is suggested by the marked increase in the glucagonolytic activity of the plasma in the presence of streptokinase. The absence of a *significant* affect of the same concentration of streptokinase on the glucagonolytic activity of the plasmas from the rat, guinea-pig, rabbit, chicken, dog and cow is in accord with the reports that the plasminogen in the plasma of these species is relatively resistant to the action of streptokinase (5, 6, 7, 8, 9, 10, 11, 12, 13, 14).

The only sub-human species whose plasma developed an increased glucagonolytic activity in the presence of streptokinase are the monkey and cat. This observation suggests that whereas the plasmas of the other sub-human species do not contain some 'proactivator' essential to the action of streptokinase, monkey and cat plasmas do contain such a factor or are more susceptible to the action of streptokinase. In accord is the demonstration by Cliffton and Downie (6) that the caseinolytic activity of monkey plasma is increased by streptokinase. No data on the influence of streptokinase on the caseinolytic or fibrinolytic activity of cat plasma is available.

Although a concentration of streptokinase which readily catalyzes the conversion of human plasminogen to plasmin is ineffective in increasing the glucagonolytic activity of the plasmas from the various sub-human species other than monkey and cat, the addition of a small amount of human plasma together with the streptokinase accelerates the activity of guinea-pig, dog and rat plasmas. Under similar conditions, however, the activity of the plasmas from rabbits, chickens and cows is not increased. Since human and other plasmas contain plasmin inhibitors, the negative results may be due to the addition of such inhibitors which in turn mask the effect of streptokinase. In accord is the demonstration that in the presence of a crude euglobulin fraction which is relatively poor in inhibitors, streptokinase produces a marked increase in the activity of the crude euglobulin fraction of the plasmas from all the sub-human species.

The preceding is in accord with the hypothesis that a human 'proactivator' may be involved in the process whereby streptokinase catalyzes the conversion of plasminogen to plasmin. The demonstration that some activation of the plasminogens from the plasmas of various sub-human species can be produced by very high concentrations of streptokinase (22, 23) does not negate this hypothesis. Nor is the hypothesis rendered invalid by the studies which suggest the activator of sub-human plasminogens is a streptokinase-plasminogen complex (15, 23) or plasmin itself (10, 12, 24). It is pertinent to note, however, that irrespective of the manner whereby streptokinase catalyzes the conversion of plasminogen to plasmin, this conversion can be produced by a variety of other 'activators' (13, 24). Among such activators are factors which are found in nearly all the tissues of various species (13, 14).

An activator of plasminogen appears rapidly in the circulation of animals and men after exposure to electroshock (25, 26, 27), the injection of pyrogens (26, 27), and the production of a local ischemia (26, 27). The release of such an activator from the tissues into the circulation is believed to be responsible for the increased fibrinolytic and/or proteolytic activity of the blood which is observed in such varied conditions as anaphylactic shock (28, 29), haemorrhagic, traumatic and thermal shock (30, 31, 32, 33, 34), acute febrile states (27, 35, 36), liver disease (37, 38, 39), toxemia of pregnancy (39), total body irradiation (40), electroshock convulsions (25, 27, 41), severe exercise (27, 42), local ischemia (27, 43), emotional tension (44, 45), after injections of adrenocorticotropin (32), adrenalin (27, 42, 46, 47), acetylcholine, 5-hydroxytryptamine, and Pituitrin® (46, 47).

The release of small amounts of endogenous activators of plasminogen may be responsible for the glucagonolytic action of plasma from apparently normal men and animals. It is doubtful that this contributes significantly to the rapid rate of destruction of glucagon by the intact animal (48, 49) or by liver extracts (50, 51, 52). Not only does the 'glucagonase' of liver extracts exhibit a more marked glucagonolytic activity but it differs from plasmin in its resistance to inhibitors of plasmin while inhibitors of 'glucagonase' do not affect the action of plasmin (53). The glucagonolytic action of plasma, however, may reflect some more general process involving plasmin since this enzyme catalyzes the hydrolysis of many proteins. Thus, in addition to its potent fibrinolytic action, plasmin catalyzes the hydrolysis of adrenocorticotropin (3, 4), somatotropin (3), serum complement (54), AC globulin (55), various plasma globulins (56), casein (57), fibrinogen (58) and other proteins. Although plasmin differs from trypsin in many respects, it resembles trypsin in its action on a number of proteins and on synthetic polypeptides containing the basic amino acids arginine and lysine (24).

The variety of situations which provoke the release of activators of plasminogen and the numerous proteins which are hydrolyzed by plasmin suggest some function in the sequence of events which characterize the somatic responses to noxious stimuli. In accord is the observation that the incubation of plasma or plasma globulins with plasmin results in the rapid production of polypeptides with vasodilator and myotrophic properties (59, 60, 61). These peptides appear to be identical with the bradykinin which is formed when plasma globulins are incubated with trypsin (61, 62). Other polypeptides which induce an increase in vascular permeability are formed under *in vitro* conditions which suggest that they are products of plasmin activity (61). The demonstration that the incubation of α-2-globulin with trypsin rapidly yields a specific tetradecapeptide which, in turn, is split by renin at its leucyl-leucine linkage to release the decapeptide angiotensin I (or hypertensin I) (63, 64) suggests that a similar sequence of events may be initiated by the action of plasmin. These and other considerations suggest the hypothesis that a variety of physiologically-active peptides are produced when plasminogen is activated in the circulation of the animal exposed to noxious stimuli. The effectiveness of such activation, however, will be dependent upon the extent to which the plasmin is inhibited by circulating inhibitors.

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THE EFFECTS OF THE ARYLSULFONYLUREAS ON THE RESPIRATORY ACTIVITY AND GLUCOSE METABOLISM OF ISOLATED RABBIT KIDNEY CORTEX[1]

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ABSTRACT

Slices of kidney cortex from fed and from fasted rabbits were incubated in the Warburg apparatus in glucose-free and glucose-containing phosphate-buffered media. The addition of 5×10^{-3} M Orinase *in vitro* effected an increase in cellular hydration and a profound reduction in oxygen consumption. Net disappearance of stable and radioactive glucose from the suspending medium was increased in five of six experiments. In all studies with 5×10^{-3} M Orinase, significantly smaller proportions of the assimilated glucose-C^{14} were incorporated into tissue lipids or recovered as radioactive CO_2. Under similar conditions, the metabolic product of Orinase, p-carboxyphenylsulfonylurea, was less than one-fifth as potent an inhibitor of the respiratory metabolism of surviving rabbit kidney cortex.

DESPITE extensive investigations designed to elucidate the mechanism of action of the hypoglycemic arylsulfonylureas, the data concerning their effects upon the respiratory metabolism of isolated cellular preparations are meager. In the present studies, surviving slices of rabbit kidney cortex were incubated in the presence of 1-butyl-3-p-tolylsulfonylurea (Orinase),[2] and 1-butyl-3-p-carboxyphenylsulfonylurea (p-carboxyphenylsulfonylurea);[2] the principal metabolic end-product of Orinase in man (1, 2). The selection of kidney tissue as the experimental preparation was prompted by several considerations. First, since Orinase and its metabolites are excreted chiefly by the kidney (3), it might be anticipated that concentrations of drug in excess of plasma levels could occur in this

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[2] Orinase is the Upjohn trademark for its brand of 1-butyl-3-p-tolylsulfonylurea. Supplies of Orinase and 1-butyl-3-p-carboxyphenylsulfonylurea (p-carboxyphenylsulfonylurea) used in these investigations were made available through the generosity of Dr. C. J. O'Donovan of Upjohn Company, Kalamazoo, Michigan.

area. Secondly, since many of the glycogenolytic, gluconeogenetic, and glucose-releasing systems which have been shown to be affected by Orinase in the liver (4, 5, 6) are also present in the kidney (7, 8, 9), it was felt that kidney tissue might afford another system for analysis of these phenomena. Finally, slices of rabbit kidney cortex constitute a hardy and metabolically-active preparation in which there is ready utilization of exogenous substrates, and in which the effects of metabolic inhibitors *in vitro* have been amply documented (10, 11).

METHODS

Seven experiments were performed. For each experiment, a male 2–3 Kg. rabbit was killed by a blow on the head. Slices of 0.3–0.4 mm. thickness were prepared from excised wedges of kidney cortex and floated on chilled saline. Occluded blood elements and plasma were removed from the slices by three successive five-minute washings with chilled saline. Slices were weighed on a torsion balance and introduced into 2.7 cc. of suspending medium contained within the main compartment of chilled Warburg vessels. The medium was composed of 0.131 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$ and 10 mM sodium phosphate buffer (pH 7.4). Experimental media were supplemented with the desired concentrations of the sodium salts of Orinase[2] or p-carboxyphenylsulfonylurea[2] and adjusted to pH 7.4 prior to the addition of phosphate buffer. The osmolarity of all media was maintained constant by appropriate reductions in the NaCl. Three separate lots of Orinase were employed: lot #11, 584-2 for experiments #1, 4, and 5; lots #11, 745-4 for experiments #2 and 3; lot #11, 715-12 for experiments #6 and 7. Only a single preparation of p-carboxyphenylsulfonylurea was available for examination. Center wells of the Warburg vessels were filled with 0.2 cc. 5% KOH and fluted strips of filter paper. Details of incubation and manometric techniques have been described previously (12). Ten minutes were allowed for gassing of flasks with pure O_2 and for thermal equilibration at 38° C. Thereafter, there was introduced from the sidearms 0.3 cc. of suspending medium containing 0.6 to 2.0 μc. of uniformly-labeled glucose-C^{14}[3] and sufficient unlabeled glucose to achieve a final glucose concentration of 196–232 mg.% in the main compartment. Following variable periods of incubation, slices were removed, weighed on a torsion balance, and quickly frozen with solid CO_2. The frozen tissues were employed for subsequent fractionation. In experiments in which the water-content of the slices was estimated, the wet slices were introduced into tared weighing bottles; wet-weight was determined immediately, and final dry-weight was measured following desiccation to constant weight in an oven at 105° C.

Aliquots of the suspending medium were analyzed for glucose by the Nelson modification of the Somogyi method (13) and precipitated as the glucosazone for radioactive assay. Center wells were rinsed and filter-paper strips were eluted with CO_2-free water. The alkaline washings and corresponding eluates were pooled. $C^{14}O_2$ was quantitatively precipiated as $BaC^{14}O_3$ with $BaCl_2$ and plated on stainless steel planchets. Extracts of total lipids were prepared by homogenizing the frozen slices with chloroform: methanol according to the method of Folch, Lees, and Sloane-Stanley (14). The extracts were washed twice to remove water-soluble contaminants and then evaporated to dryness in tared weighing vessels. The dried residues were resuspended in petroleum ether, and plated on lens paper discs contained within stainless steel planchets. All determinations were performed with duplicate vessels.

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[3] Uniformly-labeled glucose-C^{14} was prepared by Tracerlab, Inc., Boston Massachusetts. The specific activity of this preparation was 0.274 mc./mM. Homogeneity was verified in the authors' laboratory by one-dimensional paper chromatography in butanol /glacial acetic acid/H_2O and propanol/ethyl acetate/H_2O solvent systems.

A Robinson windowless proportional flow counter was employed for radioactive assays and samples were corrected for self-absorption to a mass of 5 mg. Sufficient counts were observed to reduce the probable error of the measurement to less than 3%.

Results were expressed as follows: Oxygen consumption was estimated in terms of QO_2 (cu. mm. O_2 per hour per mg. of initial wet weight). Relative changes in the mass of the suspended slices during incubation were assessed by ratios of final/initial wet weights. The net assimilation of glucose, expressed as "μg./mg. initial wet weight," was derived on the basis of chemical estimates of the glucose concentration of the media at the beginning and the end of incubation, and a known volume of 3 cc. of suspending medium within each flask. Specific activities of the glucose in the final suspending media were determined on the basis of glucosazones and calculated as "counts/mg. glucose." The formation of radioactive CO_2 and lipids from uniformly-labeled glucose was quantitated in terms of "% of the total added radioactivity."

RESULTS

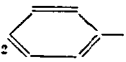
1) *Oxygen Consumption:* Results of the seven experiments are listed in Table 1. Inclusion of 5×10^{-3} M Orinase in the suspending medium effected a uniform and highly significant reduction in the oxygen consumption of surviving rabbit kidney slices. During the first hour of incubation in glucose-containing media, the QO_2 values of slices from fasted and from fed rabbits averaged 49.7% and 61.9% respectively of control values. Percentile inhibitions were even greater during the second and third hours of incubation. A comparable depression of respiratory activity by 5×10^{-3} M Orinase was observed in one experiment (#5) wherein glucose was omitted from the suspending medium. The metabolic product of Orinase, p-carboxyphenylsulfonylurea, was not as potent an inhibitor of respiratory metabolism. In three experiments, the average oxygen consumption during the first hour of incubation with $5\times10^{-}$ M p-carboxyphenylsulfonylurea was 91% of the control value. The inhibition approximated that which was observed with 1×10^{-3} M concentrations of Orinase.

2) *Slice Weight:* A significant reduction in the wet weight of slices occurred during incubation. The final wet weights of slices from control vessels in seven experiments averaged 78.8 of the initial wet weight (Table 1). Inclusion of 1×10^{-3} M Orinase or 5×10^{-3} M p-carboxyphenylsulfonylurea in the suspending medium did not affect the manifest loss of weight. However, a preservation of wet-tissue weight, and, in two instances, an actual gain in weight occurred during incubation with 5×10^{-3} M Orinase. Results of desiccation revealed that the apparent maintenance of wet weight by 5×10^{-3} M Orinase was caused by an increase in the tissue content of water (Table 2).

3) *Utilization of Glucose:* Based on chemical determinations, the net disappearance of glucose from the medium (i.e. μg. glucose/mg. initial wet weight) was increased by 5×10^{-3} M Orinase in five of six experiments (Table 3). Less pronounced effects were also produced by 1×10^{-3} M

TABLE 1. THE EFFECTS OF THE ARYLSULFONYLUREAS UPON THE RESPIRATORY METABOLISM OF SURVIVING SLICES OF RABBIT KIDNEY CORTEX

Expt. #	Diet	Initial medium glucose (mg. %)	Initial slice wt. (mg.)	Incubat. (mins.)	Drug*	Conc. (1×10^{-3} M)	QO_2†	Final wt. / Initial wt.
1	Fasted	232	200 ± 10	90	None	—	1.63	0.767
					R-CH_3	5	0.96	0.928
					R-CH_3	1	1.22	0.750
					R-CH_3	0.1	1.42	0.792
2	Fasted	212	350 ± 10	120	None	—	1.71	0.765
					R-CH_3	5	0.74	0.962
3	Fasted	196	300 ± 10	150	None	—	2.02	0.758
					R-CH_3	5	0.95	0.967
					R-COOH	5	1.83	0.722
4	Fed	221	150 ± 10	90	None	—	1.69	0.817
					R-CH_3	5	0.84	0.946
5	Fed	0	150 ± 10	90	None	—	1.40	0.755
					R-CH_3	5	0.46	0.875
6	Fed	217	300 ± 10	180	None	—	1.80	0.838
					R-CH_3	5	1.17	1.114
					R-CH_3	1	1.52	0.826
					R-COOH	5	1.58	0.838
7	Fed	198	310 ± 10	180	None	—	1.68	0.818
					R-CH_3	5	1.20	1.105
					R-CH_3	1	1.46	0.778
					R-COOH	5	1.59	0.754

* R = $CH_3CH_2CH_2CH_2NHCONHSO_2$

R-CH_3 refers to 1-butyl-3-p-tolylsulfonylurea (Orinase) and R-COOH refers to 1-butyl-3-p-carboxyphenylsulfonylurea (p-carboxyphenylsulfonylurea).

† Average values for QO_2 (μl. of O_2/mg. initial wet weight/hr.) during the first hour of incubation.

TABLE 2. THE EFFECT OF THE ARYLSULFONYLUREAS UPON THE WATER-CONTENT OF SURVIVING SLICES OF RABBIT KIDNEY CORTEX

Expt.	Drug	Conc. (1×10^{-3}M)	Slice weight (mg.)			Final water content (mg.)
			Initial	Final		
			wet	wet	dry	
3A	None	—	306.5	216.5	44.1	172.4
	R-CH_3	5	296.5	257.8	39.3	218.5
	R-COOH	5	307.5	214.3	46.0	168.3
7A	None	—	304.5	235.5	56.8	178.7
	R-CH_3	5	300.5	319.2	52.7	266.5
	R-CH_3	1	308.0	235.0	54.2	180.8
	R-COOH	5	309.5	231.2	53.2	178.0

Orinase and 5×10^{-3} M p-carboxyphenylsulfonylurea. Recalculation of results in terms of μg. glucose/mg. final wet weight reduced the magnitude but did not alter the direction of the differences between control and experimental values.

In preliminary studies with homogenates of rabbit kidney cortex, the presence of glucose-6-phosphatase activity was easily demonstrated (15). Enzyme activity per gram of tissue was increased in fasted animals. Similar dietary dependencies have recently been reported for the glucose-6-phosphatase of rat kidney (9). However, despite the enzymatic potential for the release of glucose, and in contradistinction to the published findings for surviving rat tissue (8), it has not been possible to demonstrate a measurable production of glucose during the prolonged incubation of kidney slices from fed or fasted rabbits in simple saline media (15). Nonetheless, it remained possible that such phenomena could occur in the glucose-containing systems employed in the present studies. Therefore, the specific radioactivities of the glucose in the media were examined at the end of incubation in order to assess the possibility that the manifest effects of Orinase resulted from an inhibition of glucose production. Within the limits of analytical accuracy, the final specific activities of the control

TABLE 3. THE EFFECTS OF THE ARYLSULFONYLUREAS UPON THE METABOLISM OF GLUCOSE BY SURVIVING SLICES OF RABBIT KIDNEY CORTEX

Expt.* #	Drug	Conc. (1×10^{-3}M)	Net assimilation of glucose (μg./mg.)	Final S.A. glucose C^{14} (counts/mg.)†	% added C^{14} recovered as	
					Lipid	CO_2
1	None	—	6.04	67,191	0.21	5.3
	R-CH_3	5	8.34	73,413	0.12	4.6
	R-CH_3	1	7.60	66,702	0.23	5.5
	R-CH_3	0.1	6.07	70,551	0.24	4.9
2	None	—	6.32	56,100	0.56	9.7
	R-CH_3	5	5.39	56,015	0.29	4.4
3	None	—	5.59	24,813	0.30	6.3
	R-CH_3	5	6.40	21,812	0.18	5.3
	R-COOH	5	6.07	24,224	0.31	6.6
4	None	—	6.26	60,715	0.17	7.7
	R-CH_3	5	7.70	57,202	0.13	5.1
6	None	—	5.72	62,437	0.28	7.0
	R-CH_3	5	8.47	58,886	0.19	6.7
	R-CH_3	1	8.23	57,376	0.31	8.8
	R-COOH	5	7.84	61,286	0.31	6.4
7‡	None	—	8.57			
	R-CH_3	5	11.05			
	R-CH_3	1	10.94			
	R-COOH	5	10.45			

* Numbers refer to the same experiments for which partial results are tabulated on Table 1.

† S.A. = Specific activity of glucose in suspending medium at end of incubation.

‡ Radioactive glucose was not employed in Expt. #7.

and Orinase-containing media were not significantly different (Table 3). Hence, the apparent increase in the assimilation of glucose in the presence of Orinase cannot be attributed to a depression of glucose production.

4) *The Metabolism of Glucose-C^{14}:* In the five experiments where radioactive measurements were obtained, the absolute incorporation of glucose-C^{14} into lipids was reduced an average of 39.5% below control values by 5×10^{-3} M Orinase (Table 3). Comparable effects were not obtained with 5×10^{-3} M p-carboxyphenylsulfonylurea. The inhibitory action of Orinase paralleled the depression of QO_2. The percentage of the added C^{14} which was evolved as radioactive CO_2 was also consistently reduced by 5×10^{-3} M Orinase. However, the effects were modest in those experiments in which Orinase increased the net assimilation of glucose.

To document the metabolic disposition of this glucose, the isotopic data were recalculated in terms of the percentile distribution of the radioactive glucose which disappeared from the medium (i.e. percentage of assimilated glucose C^{14}). This calculation disclosed that a significantly smaller proportion of the assimilated glucose was oxidatively decarboxylated or incorporated into lipids in the experiments where 5×10^{-3} M Orinase enhanced the manifest removal of glucose from the medium.

DISCUSSION

During the incubation of surviving slices of rabbit kidney cortex in phosphate-buffered saline and an atmosphere of oxygen, 5×10^{-3} M Orinase effected an approximate halving of oxygen consumption. At the same time, cellular water increased, an effect which has been previously described as an invariable concomitant of respiratory inhibition in isolated rabbit kidney (11). These changes in oxidative activity and cellular hydration occurred in glucose-free as well as glucose-supplemented media. Glucose-C^{14} was employed to document some of the metabolic sequelae. In all studies, the absolute incorporation of radioactivity into tissue lipids was profoundly reduced by 5×10^{-3} M Orinase. However, effects upon the utilization of glucose were more variable. In five of six experiments, 5×10^{-3} M Orinase caused a moderate apparent increase in the net disappearance of glucose from the suspending medium. Measurements of the final specific activities of the uniformly-labeled glucose in the suspending media would make it appear unlikely that this reflected merely an inhibition of renal glucose-production. An alteration in the metabolic disposition of the glucose was indicated by a uniform reduction in the percentage of the assimilated counts which appeared as radioactive CO_2 or lipid. From the data it may be inferred that an increased accumulation of such glycolytic intermediates as lactic acid must have occurred. The less pronounced effects of Orinase upon the absolute than upon the proportional production of radioactive CO_2 would also be consonant with a relative preservation of glycolytic pathways and a continued elaboration of

CO_2 *from sources other than the tricarboxylic acid cycle, such as, possibly,* the renal phosphogluconate oxidation pathway (16). These phenomena were not related to previous dietary intake since tissues from fasted as well as from fed donor animals were inhibited to a similar extent. Three separate lots of crystalline Orinase were employed for the *in vitro* observations. It would thus seem unlikely that the results can be attributed to a contaminant which is not present in the commercial, and clinically efficacious, preparations of the drug.

The effects upon oxidative metabolism were closely linked to chemical structure. Mudge has reported that the hydration and QO_2 of surviving-rabbit kidney cortex is unaffected by the substituted sulfonamides which inhibit carbonic anhydrase (11). In the present studies, p-carboxyphenylsulfonylurea, which differs from Orinase only by the substitution of a —COOH for the terminal —CH_3 group (1, 2), was less than one-fifth as potent as Orinase in depressing oxygen consumption. Although the differences between Orinase and p-carboxyphenylsulfonylurea may have resulted from variation in cellular penetration or fixation, it is of interest that the latter compound does not produce hypoglycemia in man (17).

Whether the above observations bear any relevance to the clinical action of Orinase remains to be defined. There is some precedent for the induction of hypoglycemia *in vivo* by a generalized depression of cellular respiration. Recently, Steiner and Williams have shown that the biguanide derivatives reduced blood sugar by an inhibition of electron transport at the cytochrome level (18). However, the biguanide derivatives are effective in types of diabetes which do not usually respond to Orinase (19), (cf. pancreatectomized animals; "brittle juvenile human diabetes") and, hence, it does not seem likely that the effects of Orinase are mediated in the same way. This does not preclude the possibility that Orinase may be selectively concentrated in certain organs or subcellular sites and there induce a localized histotoxic anoxia. If such were the case, the effects of Orinase to a) release performed stores of insulin (20, 21, 22), or b) retard the degradation of insulin (23), or c) diminish hepatic production of glucose (24, 4, 5, 6) could be secondary to a localized change in respiratory metabolism. It is even conceivable that a localized "Pasteur effect" induced by Orinase could cause focal changes in glucose utilization. Alternatively, the present findings may constitute an isolated toxic phenomenon which is unrelated to the therapeutic action of Orinase. The available data do not justify attempts to differentiate between these possible interpretations. It should be stressed that only one tissue from a single species was examined, and that the experimental concentrations of drug were five to ten times as great as those plasma levels which are therapeutically effective *in vivo*. Clearly, at present, more than descriptive analysis is not warranted.

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A MYOTROPHIC AGENT AND GONADOTROPHIN INHIBITOR, 19-NORTESTOSTERONE-17-BENZOATE

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ABSTRACT

19-Nortestosterone-17-benzoate, a new ester of 19-nortestosterone, has been compared with testosterone propionate for several biological activities. Testosterone propionate and 19-nortestosterone-17-benzoate are myotrophic and suppress production or release of pituitary gonadotrophins. Unlike testosterone propionate, the 19-nortestosterone derivative elicits these responses in doses which do not stimulate growth of seminal vesicles and ventral prostate in rats. The uterotrophic, anti-estrogenic and progestational potencies of the 2 androgens are similar. 19-Nortestosterone-17-benzoate is devoid of estrogenic ativity. A 6-week study in intact and castrate male and female rats demonstrate that the biologic properties of 19-nortestosterone-17-benzoate are typical of those of an androgen. This steroid was orally inactive at the dose tested.

ANDROGENIC steroids in the male, in addition to stimulating primary and secondary sex organs, are protein anabolic (1) and inhibit production of pituitary gonadotrophins (2, 3). Eisenberg and Gordan (1) demonstrated that trophic activity of androgens on the levator ani muscle of rats is a measure of protein anabolism. 19-Nortestosterone and derivatives of 19-nortestosterone have been reported to differ from testosterone propionate by being myotrophic in doses which produced no stimulation of the reproductive organs (4–7). There has been little reported on the antigonadotrophin activity of 19-nortestosterone derivatives.

In the present investigation, the biologic activities of 19-nortestosterone-17-benzoate,[3] a new ester of 19-nortestosterone, have been compared to those of testosterone propionate.

MATERIALS AND METHODS

Compounds were administered in solution or suspension in olive oil.

Myotrophic and androgenic assays. The method of Eisenberg and Gordan (1), as modified by Hershberger, Shipley, and Meyer (4), was employed. Weanling male rats weighing between 40 and 50 gm. were castrated and divided into dose groups of 5 or 10 animals each. Starting on the day following castration, the compounds were adminis-

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[3] Obtained from Organics, Inc., Chicago. Illinois.

tered once a day for 7 days. The animals were sacrificed by decapitation on the following day and seminal vesicles, ventral prostate and levator ani muscles removed and weighed. Fluid was expressed from the seminal vesicles prior to weighing.

Myotrophic and androgenic activities of 19-nortestosterone-17-benzoate and testosterone propionate were also determined in immature intact rats. The techniques employed in the intact rats were identical to those described for the castrate rats.

Uterotrophic and anti-estrogenic activities. Uterotrophic and anti-estrogenic activities were determined in 6–8 gm. immature female mice. 19-Nortestosterone-17-benzoate or testosterone propionate was administered subcutaneously twice a day for 3 days to dose groups of 5 or 10 animals, either alone or in combination with 0.3 µg. of estradiol benzoate. The animals were sacrificed on the day following the last injection. The amounts of fluid in the uteri were estimated, the uteri removed, fluid expressed, and the uteri weighed.

Estrogenic activity. The vaginal smear technique in castrate rats was employed. 19-Nortestosterone-17-benzoate was administered subcutaneously in 2 doses 8 hours apart to dose groups of 10 rats each. Vaginal smears were obtained 56, 64, and 72 hours after the first dose.

Anti-gonadotrophic activity. Gonadotrophin inhibition was measured by the parabiotic rat method of Byrnes and Meyer (8). Thirty-day-old female litter mate rats were joined in parabiosis and the right partner was ovariectomized. The compounds were administered once a day for 10 days to the castrated partner, beginning on the day of operation. The animals were sacrificed on the 11th day, the ovaries and uteri were removed and weighed.

Anti-gonadotrophic activity was also measured in immature male rats. The compounds were administered subcutaneously once daily for 10 days. The animals were sacrificed on the day following the last injection and testes, seminal vesicles, ventral prostates and levator ani were removed and weighed. The fluid was expressed from the seminal vesicles before weighing.

Progestational activity. Progestational activity was determined by the method of McPhail (9). Dose groups of 6 immature female rabbits each were administered 3 intramuscular injections of 5 µg. estradiol on alternate days. They then received 5 daily intramuscular injections of progesterone, testosterone propionate or 19-nortestosterone-17-benzoate. The rabbits were sacrificed on the day following the last injection. The uteri were weighed, sectioned and graded for degree of progestational response.

Subchronic study of 19-nortestosterone-17-benzoate. Six groups of 10 male rats and 6 groups of 10 female rats, weighing 125–150 gm. each were employed. Of these, three male groups and three female groups were castrated approximately 10 days before starting the experiment.

Two groups of castrate and intact males and females were administered 19-nortestosterone-17-benzoate subcutaneously 5 days a week for 6 weeks, in daily doses of 3 mg. /Kg. and 15 mg./Kg. Similar groups served as controls and were administered olive oil subcutaneously.

RESULTS

Myotrophic and androgenic activities. Myotrophic and androgenic activities of 19-nortestosterone-17-benzoate and testosterone propionate in castrate and in intact rats are summarized in Figure 1. The levator ani of intact and castrate rats increased in weight with smaller doses of 19-nortestosterone-17-benzoate than did either ventral prostate or seminal vesicles. The smallest dose of testosterone propionate required to produce an increase in levator ani weight also produced an increase in weight of

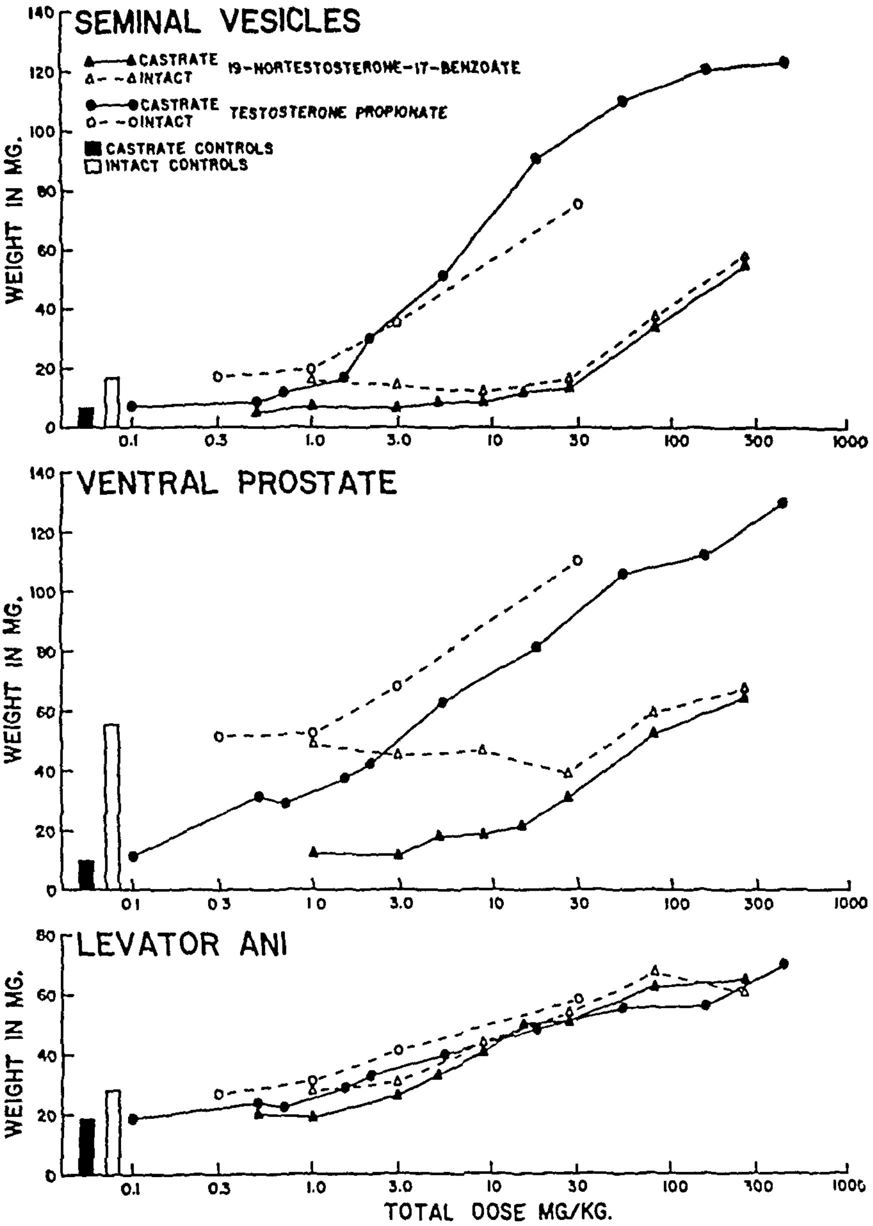

FIG. 1. Myotrophic and androgenic activity of 19-nortestosterone-17-benzoate and testosterone propionate in castrate and intact rats.

ventral prostate and seminal vesicles of intact rats and ventral prostate of castrate rats.

The dose of 19-nortestosterone-17-benzoate required to increase levator ani weight by 50% for castrate and intact animals is respectively 2 and 3 times that for testosterone propionate. The dose required to increase by 50% the weights of the seminal vesicles and ventral prostate for 19-nortestosterone-17-benzoate was much larger than that of testosterone propionate. The values ranged from 18 to 42 times that for testosterone propionate (Table 1). Oral doses of 50 mg/Kg. were ineffective in producing weight changes in levator ani and accessory sex organs.

TABLE 1. MYOTROPHIC AND ANDROGENIC DOSES (MG./KG.) IN CASTRATE AND INTACT MALE RATS PRODUCING 50% INCREASE IN ORGAN WEIGHT

Compound	Seminal vesicles		Ventral prostate		Levator ani	
	Castrate	Intact	Castrate	Intact	Castrate	Intact
19-nortestosterone-17-benzoate	13	40	4	270	2.5	8.1
Testosterone Propionate	0.7	1.4	0.12	6.4	1.2	2.8

Uterotrophic and anti-estrogenic activities. Testosterone propionate and 19-nortestosterone-17-benzoate had similar uterotrophic and estrogenic antagonism potencies in the immature mouse (Fig. 2).

Estrogenic activity. 19-Nortestosterone-17-benzoate was not estrogenic in doses ranging from 0.01 mg. to 5 mg.

Anti-gonadotrophic activity. The minimal dose for pituitary gonadotrophin suppression in the parabiotic rats was <0.01 mg. for testosterone propionate and was <0.1 mg. for 19-nortestosterone-17-benzoate (Table 2).

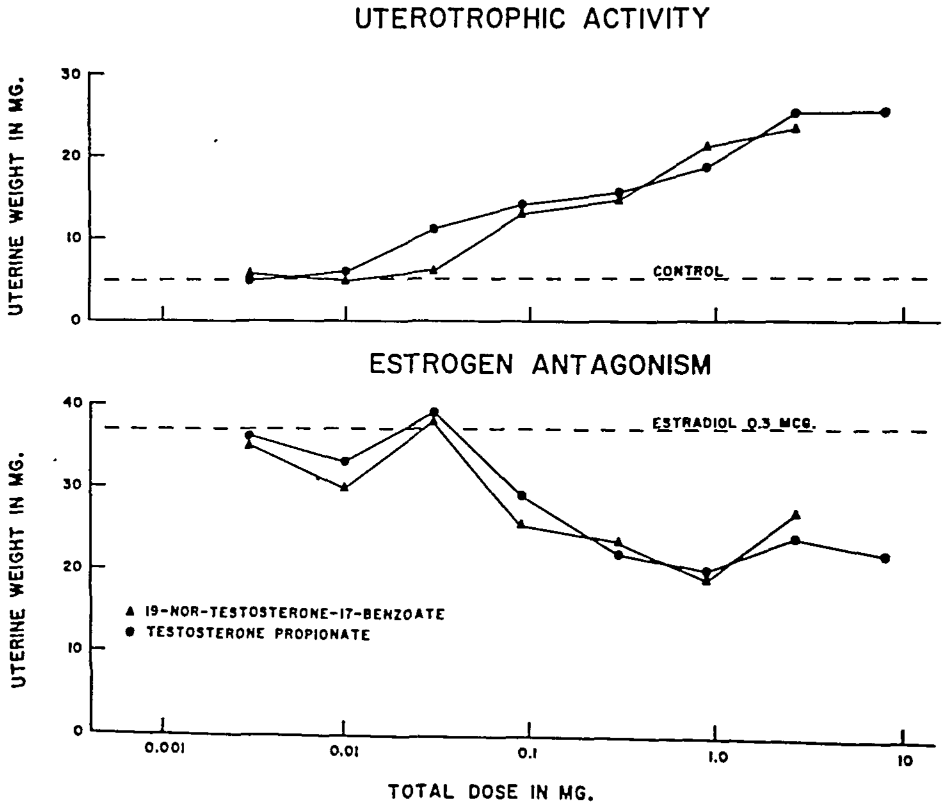

FIG. 2. Uterotrophic and anti-estrogenic activities of 19-nortestosterone-17-benzoate and testosterone propionate in immature mice.

TABLE 2. GONADOTROPHIN INHIBITION ACTIVITY OF 19-NORTESTOSTERONE-17-BENZOATE AND TESTOSTERONE PROPIONATE IN PARABIOTIC FEMALE RATS

Treatment	Daily dose, mg.	Castrate uterus, mg.	Intact uterus, mg.	Intact ovaries, mg.
Control		26	147	125
Testosterone Propionate	1.0	154	44	16
Testosterone Propionate	0.1	37	29	20
Testosterone Propionate	0.01	32	93	43
Testosterone Propionate	0.001	62	210	143
19-nortestosterone-17-benzoate	1.0	156	35	16
19-nortestosterone-17-benzoate	0.1	31	41	21
19-nortestosterone-17-benzoate	0.01	49	167	119

In mature male rats, 19-nortestosterone-17-benzoate was gonadotrophic inhibitory and myotrophic in doses which were not androgenic (Table 3). Testis weights of animals administered 0.5 mg./Kg. and 2.5 mg./Kg. were lower and levator ani weights were greater than those of controls. The seminal vesicles and ventral prostates weighed about the same or less than those of controls. Testosterone propionate in daily doses of 0.25 mg./Kg. and 1.25 mg./Kg. had effects on testes and levator ani similar to that of 19-nortestosterone-17-benzoate. It was more androgenic, however, since it increased the weights of ventral prostate and seminal vesicles.

Progestational activity. 19-Nortestosterone-17-benzoate and testosterone propionate had similar progestational activity (Table 4). The progestational potency of these steroids was much lower than that of progesterone.

Subchronic study of 19-nortestosterone-17-benzoate. Food consumption of intact female rats administered 19-nortestosterone-17-benzoate in doses of 3 mg./Kg. per day and 15 mg./Kg. per day and castrate female rats administered 3 mg./Kg. per day was greater than that of controls or the other experimental female group and they gained more weight. Food consumption and body weight changes for intact and castrate male rats administered the steroid were similar to that for controls. Hematologic values were similar for experimental and control groups. Organ weights (Tables

TABLE 3. EFFECTS OF 19-NORTESTOSTERONE-17-BENZOATE AND TESTOSTERONE PROPIONATE IN INTACT MALE RATS AFTER 10 DAILY SUBCUTANEOUS INJECTIONS

Treatment	Daily dose, mg./Kg.	Body wt. gain, gm.	Daily food intake, gm./rat	Organ weight, mg.				
				Pituitary	Seminal vesicles	Ventral prostate	Levator ani	Testes
Control (Olive oil)		46	10.1	4.1±0.3*	16.2±2.1	45.7± 5.8	30.5±3.5	1540± 82
19-nortestosterone-17-benzoate	2.5	56	11.2	4.2±0.3	15.0±0.3	27.7± 3.6	68.6±7.0	715±107
19-nortestosterone-17-benzoate	0.5	48	10.4	3.8±0.1	11.2±1.3	30.0± 1.3	40.5±1.4	880± 88
Testosterone propionate	1.25	56	11.0	4.4±0.1	136.2±9.4	151.1±12.5	73.6±2.9	721± 53
Testosterone propionate	0.25	48	10.6	5.0±0.4	32.3±2.1	57.3±10.5	41.0±1.7	982± 88

* Standard error of the mean.

5 and 6) demonstrate that 19-nortestosterone-17-benzoate prevented castration hypertrophy of the pituitary and did not significantly alter thyroid or liver weight. The adrenal weight in intact and castrate females and castrate males and the thymus weight of all rats administered 19-

TABLE 4. PROGESTATIONAL ACTIVITY OF 19-NORTESTOSTERONE-17-BENZOATE AND TESTOSTERONE PROPIONATE IN THE IMMATURE FEMALE RABBIT

Treatment*	Total dose, mg.	Uterine wt., gm.	Uterine histology†
Estrogen Primed control	—	1.14	6L
Progesterone	0.75	2.35	6H
Testosterone Propionate	27.0	2.59	6M
Testosterone Propionate	9.0	1.60	5M, 1L
Testosterone Propionate	3.0	1.25	6L
Testosterone Propionate	1.0	1.06	5L
19-nortestosterone-17-benzoate	27.0	2.42	6M
19-nortestosterone-17-benzoate	9.0	2.46	2M, 4L
19-nortestosterone-17-benzoate	3.0	1.40	6L
19-nortestosterone-17-benzoate	1.0	1.19	6L

* All animals were pretreated with 15 μg. estradiol monobenzoate.
† Number preceding letter indicates number of animals.
H = Typical progestational uterus.
M = Moderate glandular proliferation.
L = Typical estrogenized uterus; flattened glands and slight invagination.

TABLE 5. BODY AND ORGAN WEIGHTS OF MALE RATS ADMINISTERED 19-NORTESTOSTERONE-17-BENZOATE FOR 6 WEEKS

	Intact males			Castrate males		
Daily Dose, mg./Kg.	15	3	—	15	3	—
Body wt.						
Initial, gm.	139	139	128	126	119	129
Final, gm.	244	284	271	231	248	253
Pituitary, mg.	7.5± 0.4*	8.5± 0.9	7.4± 0.4	7.4± 0.5	8.6± 0.7	12.0± 0.4
Thyroid, mg.	13.0± 0.8	11.2± 1.1	11.3± 0.7	11.0± 0.7	11.4± 1.1	10.8± 1.0
Adrenals, mg.	34.3± 2.3	34.0± 2.4	32.2± 2.1	31.4± 1.2	29.8± 1.6	44.3± 2.8
Thymus, mg.	140.0±11.0	234.0±27.0	384.0±22.0	145.0±43.0	229.0±21.0	513.0±27.0
Heart, mg.	858.0±29.0	879.0±22.0	861.0±32.0	821.0±22.0	825.0±26.0	801.0±26.0
Liver, gm	8.5± 0.3	10.6± 0.6	10.1± 0.4	7.7± 0.8	8.4± 0.3	9.5± 0.5
Kidneys, gm.	2.2± 0.1	2.1± 0.1	1.8± 0.1	2.0± 0.1	1.9± 0.1	1.7± 0.1
Testes, gm.	2.7± 0.1	1.7± 0.1	2.7± 0.1			
Seminal Vesicles, mg.	365.0±17.0	88.0± 8.0	228.0±30.0	341.0±18.0	89.0± 8.0	15.0± 1.0
Ventral Prostate, mg.	386.0±19.0	79.0±23.0	290.0±46.0	392.0±29.0	44.0± 5.0	11.0± 1.0
Levator Ani, mg.	278.0±15.0	270.0±10.0	187.0±16.0	220.0±12.0	220.0±13.0	46.0± 4.0

* Standard error of the mean.

TABLE 6. BODY AND ORGAN WEIGHTS OF FEMALE RATS ADMINISTERED 19-NORTESTOSTERONE-17-BENZOATE FOR 6 WEEKS

	Intact females			Castrate females		
Daily Dose, mg./Kg.	15	3	—	15	3	—
Body Wt.						
Initial, gm.	121	121	115	121	132	131
Final, gm.	231	234	187	229	258	231
Pituitary, mg.	8.0± 0.5*	8.2± 0.5	9.8± 0.6	9.4± 0.3	9.4± 0.4	13.5± 0.6
Thyroid, mg.	14.8± 0.8	14.8± 0.9	13.9± 0.6	15.8± 1.2	15.3± 0.5	14.9± 0.5
Adrenals, mg.	34.5± 1.5	34.5± 1.5	50.6± 4.0	35.2± 1.6	29.2± 1.3	50.4± 3.6
Thymus, mg.	89.3± 6.1	228.0±26.0	308.0±24.0	129.0±14.0	233.0±16.0	504.0±38.0
Heart, mg.	769.0± 31.0	788.0±28.0	659.0±23.0	840.0±24.0	844.0±29.0	735.0±24.0
Liver, gm.	7.4± 0.4	8.7± 0.4	6.9± 0.2	8.2± 0.4	9.0± 0.2	7.3± 0.1
Kidneys, gm.	1.8± 0.1	1.8± 0.1	1.4± 0.1	1.9± 0.1	1.9± 0.1	1.5± 0.1
Ovaries, mg.	18.7± 1.1	25.8± 2.7	56.8± 3.6			
Uterus, mg.	691.0±124.0	454.0±26	288.0±25.0	326.0±21.0	312.0±12.0	45.0± 3.0

* Standard error of the mean.

nortestosterone-17-benzoate were lower than those of the control rats. Treatment with this steroid resulted in larger kidney weights in all animals and in increased heart weights in all females. Ovarian weights were lower and uterine weights were higher than those of the control animals. Testes, seminal vesicles, and ventral prostate weights were reduced with the lower dose of the drug only.

DISCUSSION

Biological studies indicate that 19-nortestosterone-17-benzoate possesses all the activities of an androgen although end organ dose response relationships do not always follow those for testosterone propionate. The minimum dose of testosterone propionate for effecting an increase in levator ani weight in the rat also increases the seminal vesicles and ventral prostate weights. In contrast, the minimum dose of 19-nortestosterone-17-benzoate for increasing the weight of levator ani muscle does not increase accessory sex organ weights. This steroid, therefore, is similar to other 19-nortestosterone derivatives in that it produces a myotrophic effect at doses which do not increase accessory sex organ weights.

Production or release of pituitary gonadotrophins are inhibited in the male rate with doses of 19-nortestosterone-17-benzoate which do not stimulate either the ventral prostate or the seminal vesicles. Non-uterotrophic levels of this steroid almost completely suppress secretion of pituitary gonadotrophins in parabiotic female rats. These studies suggest that the pituitary is very sensitive to this compound. The anti-gonadotrophic action of 19-nortestosterone-17-benzoate can be assumed to be a property of its androgenicity since this steroid was not estrogenic in the castrate female rat.

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ENHANCING EFFECT OF CORTICOTROPHIN ON THYROTROPHIN PRODUCED EXOPHTHALMOS IN FISH

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ABSTRACT

Goldfish (*Carassius auratus*, var. *japonicus*, subvar. *bicaudatus*) respond to the administration of thyrotrophic hormone with exophthalmos. Eye protrusion increases with simultaneous injections of corticotrophin. Retrobulbar injections of normal saline, human serum or pituitary hormones produce a transient exophthalmos, which is much more persistent when thyrotrophin is injected. Previous treatment of fishes with long-acting corticotrophin prolonged the duration of the exophthalmos caused by retrobulbar injections of thyrotrophin; this effect is not observed when other fluids are injected.

SOME fish respond with an increase in the protrusion of the ocular globes as a result of different hormonal stimuli. Albert (2) produced exophthalmos in *Fundulus heteroclitus* by means of intracelomic injections of anterior pituitary extracts. Dobyns and Steelman (6) showed the possibility of obtaining the same effect by injecting preparations of pituitary glands free from thyrotrophic activity. Pickford (10), on the contrary, observed that there is a strict relation between the exophthalmos-producing and thyroid-stimulating effects of thyrotrophic preparations. Exophthalmos was also observed in common minnows in response to treatment with triiodothyronine and desiccated thyroid (8). On the other hand, Matty and co-workers (9) succeeded in producing exophthalmos by injecting androgens in two species of teleost fish (*Sparisoma squalidum* and *Scarus croicensis*) and by the injection of thyroxine and triiodothyronine in these species and in *Bathystoma aurolineatum*.

In Spain it is impossible to obtain *Fundulus heteroclitus* or any of the species of fish with which the authors mentioned above have worked. In a series of previous tests we investigated the possible exophthalmic action of anterior pituitary extracts in the following fish: *Barbus fluviatilis*, *Cyprinus carpio*, *Leuciscus crithrophthalmus*, *Fundulus hispanicus*, *Carassius auratus*, *Perca fluviatilis*, *Tinca tinca*, *Gobitis fossilis*, *Gobius fluviatilis* and *Amciurus nebulosus*. All these, except the last, under diverse experimental conditions can develop exophthalmos following injections of thyrotrophin.

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Nevertheless we were only able to obtain *Carassius auratus* var. *japonicus* in unlimited quantities and we used this species for almost all our investigations. Between the diverse types of goldfish, the *Carassius auratus* var. *japonicus* subvar. *bicaudatus* show a greater sensitivity and uniformity of response. This subvariety has a more globular body and a flatter head. *Carassius macrophthalmus* has been derived from this fish by selection; in this species protrusion of the ocular globes, which is sometimes enormous, is an inherited characteristic (Fig. 1).

We have presented numerous clinical and experimental arguments in previous papers (4, 5) supporting the view that the exophthalmos of Graves' disease and other related forms of ophthalmopathy (oedematous exophthalmos, malignant exophthalmos, infiltrative ophthalmopathy, exophthalmic ophthalmoplegia, etc.) are secondary to a hyperfunction of the anterior pituitary. According to Aterman (3) the adrenal cortex also participates in the pathogenesis of these conditions. Experimenting with golden hamsters we have confirmed that the presence of adrenal glands is indispensable in the production of exophthalmos by injection of thyrotrophin and that previous treatment with corticotrophin or adrenal steroids reinforces the exophthalmic action of the thyrotrophic hormone (5).

FIG. 1. Telescopic-eyed goldfish (*Carassius macrophthalmus*) with genetic constitutional exophthalmos and a common goldfish (*Carassius auratus*).

The series of experiments that follows was planned to study the effect of corticotrophin on the exophthalmos produced in *Carassius auratus* var. *japonicus*, subvar. *bicaudatus* by intracoelomic injections of thyrotrophin and its influence on ocular protrusion following the injection of thyrotrophin directly into the retrobulbar tissues.

METHODS

Groups of ten *Carassius*, about two years old (9–10 cm. long) were injected intracoelomically at intervals of eight hours with thyrotrophic extract, the doses varying from 0.25 to 25.00 Heyl-Laqueur units. Other similar groups were injected with corticotrophin, chorionic gonadotrophin, growth hormone, human serum and normal saline solution. The treatment was continued for 5 consecutive days, during which the appearance of the fish was recorded and the protrusion of ocular globes measured with a caliper with vernier scale.

Other groups of goldfish were treated with the same doses of thyrotrophin but adding to each injection two international units of corticotrophin. Control groups were similarly treated with corticotrophin and chorionic gonadotrophin, growth hormone extract, human serum and normal saline solution.

In another experiment the exophthalmic effects of the retrobulbar injections of thyrotrophin were studied. The material was injected only once through the conjunctival sac of both eyes with an insulin syringe and a needle of small caliber. The dose was 0.05 cc. (5 u. Heyl-Laqueur). Control fish were injected with the same volume of corticotrophin, chorionic gonadotrophin, growth hormone and normal saline solution. The resulting ocular protrusion was measured immediately after the injections and at regular intervals of 8 hours.

Other fish were previously treated intracoelomically with a dose of 5 I.U. of corticotrophin-zinc following which exophthalmos was brought about in the same manner as in the preceding experiment.

RESULTS

The intracoelomic injections of thyrotrophin at doses of 10 u. Heyl-Laqueur (total quantity injected 150 u.) produced a variable exophthalmic effect. Only in two of ten injected fish was a small augmentation of the ocular protrusion and the intercorneal distance observed (1.5 mm. and 1.8 mm. respectively). Administring 25 u. by injection (total dose 375 u.), exophthalmos was observed in seven fish, in four of which it was perfectly evident on simple inspection and without the necessity of measuring intercorneal distance (Fig. 2). The increase in the intercorneal distance was 3.8 mm. (±1.15 mm.). Doses greater than 25 u. were poorly tolerated and doses of less than 10 u. had no effect. Exophthalmos was not observed in any of the fish in the control groups injected with corticotrophin, growth hormone, chorionic gonadotrophin, human serum or normal saline solution.

In the animals showing positive results, the exophthalmos began to appear on the third day of the experiment, became more evident in the two following days and disappeared completely at the end of two or three days after ceasing the injections of thyrotrophin.

We observed that the injection of corticotrophin reinforced the exophthalmic effect of thyrotrophin without increasing the intensity of the maximum result. Injections of 10 u. of thyrotrophin, with a very small exophthalmic effect (see above), produced exophthalmos in six fish with increase of the intercorneal distance of 2.9 mm. (±0.6 mm.) when it was injected with corticotrophin. Injections of 25 u. of thyrotrophin caused exophthalmos in nine fish. The evolution of exophthalmos followed the same course as in the animals injected only with thyrotrophin without complementary treatment with corticotrophin. Control groups injected with thyrotrophin and chorionic gonadotrophin, growth hormone, human serum or normal saline solution developed exophthalmos to the same degree as the fish treated with thyrotrophic hormone alone. These materials are devoid of the enhancing effect of corticotrophin.

The retrobulbar injections of a small quantity of normal saline produced a transitory increase of ocular protrusion. An augmentation of 5 mm. or more in the intercorneal distance could thus be obtained without ill effects. This exophthalmos persisted for some four hours, then diminished progressively and disappeared in the course of the following eight hours. Injection of gonadotrophic hormone, growth hormone or human serum produced the same phenomenon, but, possibly because of the protein composition of these fluids, the exophthalmos persisted for about 24 hours. On the contrary, the exophthalmos produced by injection of thyrotrophin was always more persistent, and after 24 hours the increase of the intercorneal distance was still 80% (±6%) of the initial value and after 48 hours it was 12% (±7%).

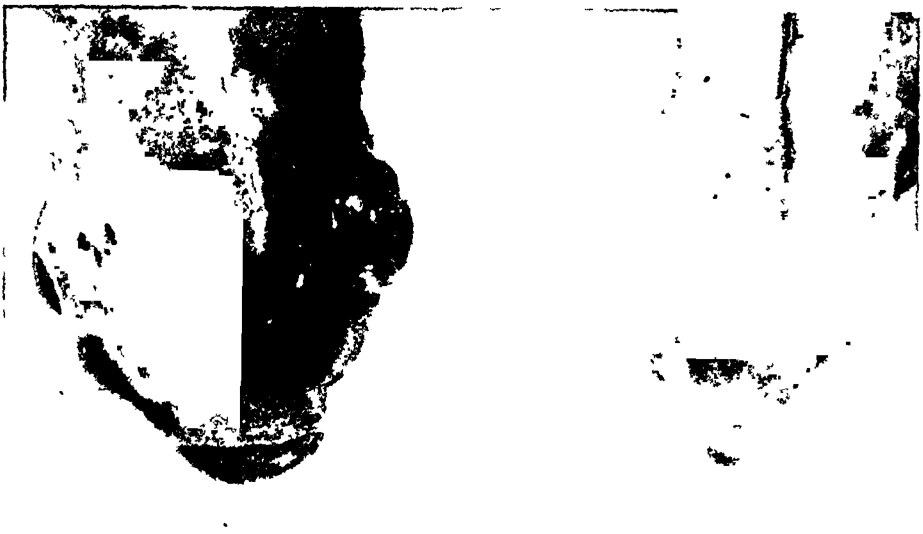

FIG. 2. Exopthalmos produced with systemic treatment with thyrotrophin. At right control fish.

In the fish treated previously with corticotrophin-zinc we observed that the increase of the ocular protrusion produced by thyrotrophin was much more persistent. After 24 hours the increase in the intercorneal distance still equaled 82% (±3%) of the initial value, after 48 hours it was 58% (±5%) and after 72 hours it was 22% (±10.5%) (Fig. 3).

FIG. 3. Ocular protrusion provoked by retrobulbar injections of thryotrophin 24 hours before. The first fish on the left was previously treated with long acting corticotrophin; the middle fish did not receive corticotrophin; on the right is the control fish in which exophthalmos was produced by injecting human serum. Note the persistence of ocular protrusion in both fish treated with thyrotrophic hormone and the larger exophthalmos in the fish that received corticotrophin.

DISCUSSION

The difficulties in producing exophthalmos in some fish by injection of anterior pituitary extracts or near pure thyrotrophic preparations suggests the existence of an exophthalmic factor distinct from the thyrotrophic hormone (1, 6, 7). Nevertheless, even though it may be unjustified to apply the results of our experiments directly as a basic explanation of the pathogenesis of the exophthalmos of Graves' disease, they confirm our hypothesis (5) that this phenomenon probably develops as a result of a combination of several endocrine factors. The action of thyrotrophic hormone is the essential causative factor; the corticotrophin and the activity of the adrenal cortex have a potentiating effect, possibly by increasing the sensitivity of the retrobulbar tissues to the exophthalmogenic action of thyrotrophin. The experiments of Langford (8) and of Matty *et al.* (9) in which they showed the possibility of producing exophthalmos in fish by thyroid hormones seem to oppose the thyrotrophic hypothesis, since the administration of thyroxine produces an inhibiting effect on the pituitary secretion of thyrotrophin. However, in previous experiments (5) we observed that large doses of thyroid hormone increase the thyrotrophic exophthalmos, whilst weak doses of the same are capable of reducing it. These results—apparently paradoxical—could be explained by the toxic action of the thyroxine, which is capable of stimulating the anterior pituitary-adrenal system (11), increases the corticotrophic and corticoid secretion, and indirectly creates experimental conditions analogous to those obtained by injecting thyrotrophin and corticotrophin.

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GROWTH-PROMOTING ACTIVITY OF PREGNANT RAT PLASMA AFTER HYPOPHYSECTOMY AND AFTER THYROIDECTOMY[1]

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ABSTRACT

Growth promoting potency of rat blood plasma, as evaluated by the tibial assay, decreased after thyroidectomy, and increased during normal pregnancy. Thyroidectomy did not interfere with the increased level during pregnancy. Hypophysectomy of the pregnant rat did not reduce the growth promoting activity of plasma to the extent which it did in non-pregnant rats.

Thyrotrophic activity (assayed by I^{131} uptake and thyroid morphology of hypophysectomized recipients) was demonstrable in plasma from both pregnant and non-pregnant thyroidectomized rats. Similar volumes of plasma from intact pregnant or non-pregnant rats elicited no response in the thyroids of recipients. Adrenocorticotrophic activity was not detected at the doses injected (by weight and morphology of the adrenals of recipients) in plasma from any type of donor (normal, thyroidectomized, hypophysectomized rats, pregnant or non-pregnant). Gonadotrophic activity was not detected in plasma of any type of donor, as judged by ovarian morphology of recipient hypophysectomized rats. However, judged by the follicular development in the donors, follicle-stimulating substances are present in plasma late in pregnancy both in normal-pregnant and thyroidectomized-pregnant donors, but not in hypophysectomized-pregnant donors. Luteotrophic substances were present both in hypophysectomized-pregnant and thyroidectomized-pregnant rats, as judged by maintenance of the corpora lutea in these donors.

GROWTH-PROMOTING activity has been reported in retroplacental blood plasma of women (1, 2) and more recently in blood plasma of pregnant rats (3, 4). As both hypophysectomy and thyroidectomy in the rat interfere with body growth, and as thyroidectomy has been found to decrease the growth-promoting activity both in the pituitary and in blood plasma (5, 6), any attempt to characterize the growth-promoting capacity of the plasma of pregnant animals, and trace the origin of this activity, would naturally be directed first toward the roles of the pituitary and the thyroid. It was the purpose of this study to determine the effect of hypophysectomy and of thyroidectomy on the growth-promoting potency of plasma in pregnant rats.

METHODS

Preparation of donors of plasma. Rats maintained on an adequate diet[2] were bred

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[2] All normal and pregnant rats, operated and non-operated, were maintained on a

when 90–120 days of age.[3] In order to determine the role of the thyroid, rats were thyroidectomized on day 1, 6 or 14 of pregnancy.[4] The blood plasma of normal-pregnant and of thyroidectomized-pregnant rats was bioassayed at intervals during pregnancy for growth-promoting activity. Other pituitary-like potencies of the plasma were determined simultaneoulsy. In order to determine the contribution of the pituitary to the growth-promoting activity of plasma, rats were hypophysectomized on day 12 of pregnancy and the plasma was bioassayed 4 or 8 days later. Plasma from non-pregnant normal and thyroidectomized rats of the same age and postoperative period was assayed simultaneously. The blood for bioassay was collected from the aorta and immediately centrifuged for 12 minutes, at 2000 rpm, under cover of mineral oil. The plasma was then aspirated into a syringe for injection. The time between withdrawal and injection was less than 30 minutes.

All donors were injected with 0.5 μc. of I^{131} 16–18 hours before autopsy. The thyroids from normal and hypophysectomized donors, and all tissues from the ventral neck region of thyroidectomized rats, were placed in Bouin's fixative to be counted in the scintillation counter (7). An I^{131} uptake in thyroidectomized rats greater than 0.05% of the injected dose, or 0.2% of the normal uptake, was considered evidence of incomplete removal of the thyroid, and the plasma from such animals was not used for bioassay. All pituitary target organs from donors were weighed and prepared for histological analysis of the endocrine status. Completeness of hypophysectomy was judged by examination of the pituitary site, by the weight of the pituitary target organs and by the I^{131} uptake of the thyroid. Only completely hypophysectomized rats were used as donors. Subsequently the completeness of the hypophysectomy was verified by histological analysis of the target organs.

Preparation of recipients. The animals used for assay of plasma were immature female rats of the Long-Evans strain, hypophysectomized at 26–28 days of age. They were injected when 2 weeks post-operative unless otherwise specified. Groups of these rats were injected with plasma from normal, thyroidectomized, pregnant, thyroidectomized-pregnant or hypophysectomized-pregnant rats. The doses of plasma varied from 1 to 5 cc. and were injected daily intraperitoneally for 4, 14 or 15 days. At least 4 rats were used in the assay of each dose of plasma, and each dose level was reassayed one or more times. Half of the controls were injected with comparable volumes of saline, but as no difference was observed between these and uninjected controls the values have been presented together. Body weight changes during the injection period were recorded in all recipients. Body growth was also evaluated in the rats injected for the longer periods from measurements of tail length at the beginning and the end of the experimental period. At autopsy the tibias were dissected, split and stained with silver nitrate, and the width of the uncalcified portion of the proximal epiphyseal cartilage was measured as evidence of growth stimulation (8, 9). The hypophysectomized recipients were injected

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modified McCollum Diet I, the diet used in the breeding colony, which consists of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate), 1% KI solution added to provide 1 μg. of I per gram of Diet. The hypophysectomized recipients were given daily a wet mash of the diet in addition to the dry diet. All rats received a supplement of lettuce 3 times weekly. The colony was maintained at 74 ± 1° F.

[3] Female rats of the Long-Evans strain, at the proestrous stage, were caged individually overnight with males. The next morning the vaginal contents were checked and if plug and sperm were present this day was considered day 0 of pregnancy.

[4] Thyroidectomy was performed surgically, the parathyroids being removed simultaneously. Hypophysectomy was performed by the usual parapharyngeal approach.

TABLE 1. ENDOCRINE STATUS OF DONORS OF PLASMA

Group		No. of rats	Day of pregnancy		Period P.O.	Ovaries weight, mg.	Adrenals weight, mg.	Thyroid* I^{131} uptake %
			Operation	Autopsy				
Normal	Non-pregnant	35	—	—	—	64	60	14.7
	Pregnant	11	—	12	—	64	64	—
		11	—	13	—	67	56	10.0
		28	—	20	—	84	64	5.7
Hypophysectomized	Non-pregnant	9	—	—	8	31	26	0.78
	Pregnant	12	12	20	8	72	34	1.35
Thyroidectomized	Non-pregnant		—	—	6	72		0.008
		12	—	—	13	57	46	0.026
		10	—	—	14	56	45	0.015
		22	—	—	20	43	38	0.012
	Pregnant	9	6	12	6	61	61	0.005
		14	1	13	12	61	46	0.026
		11	6	20	14	89	65	0.010
		15	14	20	6	80	52	0.013
		8	1	20	19	87	47	0.025

* The values for I^{131} uptake were determined on the same day and are therefore not attributable to differences in dietary iodine intake.

with 0.5 μc. of I^{131} sixteen hours before autopsy. Their thyroids were fixed and counted in a scintillation counter, and later were sectioned and stained with hematoxylin and eosin. The thymus was dissected and weighed. The completeness of hypophysectomy of the recipients was judged by the same criteria as those used for donors.

RESULTS

The endocrine status of the donors of plasma is indicated by the data in Fig. 1 and Table 1. The normal-pregnant rats increased in body weight 98 gm. by day 20 of pregnancy (Fig. 1). The hypophysectomized-pregnant rats increased in weight by approximately the same amount by day 20 (94 gm.). Thyroidectomized-pregnant rats gained somewhat less by this

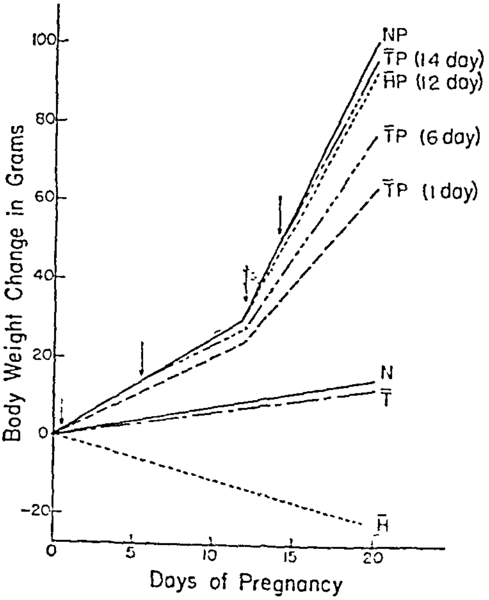

FIG. 1. Graph showing body weight gain of rats in the following categories: normal-pregnant (NP) thyroidectomized-pregnant ($\bar{T}$P) with operation performed on different days of pregnancy, as indicated by the arrows and the figures in parentheses, and hypophysectomized-pregnant ($\bar{H}$P), operation day 12 of pregnancy, as compared with appropriate non-pregnant controls: normal (N), thyroidectomized ($\bar{T}$) or hypophysectomized ($\bar{H}$); during pregnancy or a comparable 20 day period.

TABLE 2. INFLUENCE OF THYROIDECTOMY ON THE GROWTH PROMOTING ACTIVITY OF THE PLASMA OF PREGNANT RATS

Donors of plasma				Hypophysectomized recipients—14 days post-operative					
Type		Day of pregnancy	Days P.O.	No. of rats	Daily dose, 4 days	Epiphyseal cartilage width	Body weight increase	Thyroid	
								I^{131} uptake	Histology
					cc.	micra*	gm.	%	
Normal	Non-pregnant	—	—	15	3.0	201 ± 2	2.2 ± 0.4	0.45 ± .08	—
		—	—	8	3.5	202 ± 5	2.3 ± 0.3	0.63 ± .09	—
		—	—	4	4.5	211 ± 5	2.8 ± 0.8	0.65 ± .10	—
	Pregnant	12	—	5	5.1	233 ± 4	7.0 ± 0.7	0.56 ± .18	—
		13	—	8	3.5	229 ± 4	5.9 ± 0.4	0.60 ± .12	—
		20	—	8	3.0	230 ± 4	3.0 ± 0.5	0.52 ± .15	—
		20	—	4	3.7	239 ± 3	2.0 ± 0.6	0.72 ± .24	—
		20	—	3	4.4	218 ± 3	4.0 ± 0.8	0.66 ± .08	—
Thyroidectomized	Non-pregnant	—	12	4	3.0	190 ± 3	3.0 ± 0.4	1.87 ± .18	+
		—	13	8	3.0	182 ± 2	2.6 ± 0.5	1.90 ± .12	+
		—	14	4	2.3	179 ± 4	2.2 ± 0.5	1.46 ± .29	+
		—	21	5	3.4	180 ± 3	2.6 ± 0.4	2.88 ± .11	++
	Pregnant	12	6	4	3.6	224 ± 6	6.5 ± 1.5	1.54 ± .27	+
		13	13	8	3.5	233 ± 7	4.3 ± 0.9	1.68 ± .12	+
		20	6	4	4.8	220 ± 4	3.5 ± 0.7	1.04 ± .07	±
		20	8	6	2.8	221 ± 3	1.0 ± 0.4	0.94 ± .03	−
		20	13	4	4.5	235 ± 6	3.5 ± 0.4	1.51 ± .18	+
		20	14	4	2.6	213 ± 5	5.0 ± 1.0		
		20	14	4	3.8	237 ± 3	2.0 ± 0.3	0.92 ± .14	−
		20	20	4	2.6	221 ± 9	2.5 ± 1.0	1.36 ± .19	−
		21	21	4	3.0	244 ± 7	5.3 ± 1.0	1.34 ± .13	±
—	—	—	—	60	0	151 ± 2	1.5 ± 0.2	0.45 ± .03	−

* $SE = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

time of pregnancy (63–90 gm.) depending on the day of pregnancy when the operation was performed; those thyroidectomized on day 1 of pregnancy gained the least. Non-pregnant thyroidectomized rats of the same age gained 13 gm. in this early post-operative period, essentially the same gain as made by normal rats during the interval (15 gm.). Hypophysectomized rats lost weight (20 gm.).

The ovarian weights of the normal pregnant rats at day 13 of pregnancy were still within the range for normal rats, but by day 20 had increased from 64 mg. to 84 mg. (Table 1). This increase in ovarian weights late in pregnancy also occurred in thyroidectomized-pregnant rats, whereas in thyroidectomized rats they decreased within the same post-operative period. Hypophysectomy on day 12 of pregnancy did not result in atrophy of the ovaries by day 20 of pregnancy, whereas in non-pregnant rats ovarian atrophy followed hypophysectomy within this interval.

The adrenal weights in normal pregnant rats remained within normal range. Adrenal weights decreased after thyroidectomy in pregnant as in non-pregnant rats. After hypophysectomy the adrenals regressed to the same extent in pregnant as in non-pregnant rats.

The I^{131} uptake of the thyroids of the normal-pregnant rats on day 13 was lower than in non-pregnant rats and was reduced further by day 20. The I^{131} uptake was markedly reduced following hypophysectomy, but after hypophysectomy in pregnant rats it remained somewhat above the level in hypophysectomized controls after the same post-operative in-

TABLE 3. INFLUENCE OF HYPOPHYSECTOMY ON GROWTH PROMOTING ACTIVITY OF PLASMA AS JUDGED BY TIBIAL ASSAY

Donors of plasma				Hypophysectomized recipients—14 day post-operative			
Type	Day of pregnancy	Days P.O.	No. of rats	Daily dose 4 days	Epiphyseal cartilage width	Body weight increase	Thymus weight
				cc.	micra	gm.	mg.
Normal	—	—	4	3.0	196 ±3	3.3 ±0.7	—
	—	—	4	3.5	198 ±4	3.2 ±0.6	155 ±6
	—	—	8	4.0	212 ±3	4.0 ±0.4	160 ±5
Normal pregnant	16	—	6	2.8	217 ±5	3.0 ±0.5	176 ±6
	20	—	4	3.0	220 ±3	5.8 ±0.6	158 ±7
	20	—	4	3.6	245 ±4	2.0 ±0.3	164 ±4
	20	—	4	5.0	226 ±2	5.5 ±1.0	151 ±5
Hypophysectomized pregnant	16	4	4	3.5	225 ±1	2.3 ±0.4	177 ±5
	20	8	8	3.0	210 ±2	1.0 ±0.1	152 ±4
	20	8	16	3.6	230 ±3	3.3 ±0.4	189 ±3
	20	8	8	4.5	225 ±2	3.5 ±0.5	168 ±4
—	—	—	60	0	151 ±2	1.5 ±0.2	110 ±3

terval. The I^{131} uptake was very low in thyroidectomized rats, whether pregnant or not, the low value being the basis of selection of donors.

Table 2 shows the influence of thyroidectomy on growth-promoting potency of plasma in pregnant and non-pregnant rats, as judged by the tibial assay. The potency of plasma of thyroidectomized rats was low, probably lower than in normal rats, whereas in thyroidectomized-pregnant rats it was higher than normal. The plasma from thyroidectomized-pregnant donors, though drawn at varying periods after thyroidectomy, contained the same amount of growth-promoting activity as that from normal-pregnant rats at the same period in pregnancy.

The thyrotrophic activity in plasma of thyroidectomized donors, both pregnant and non-pregnant, was increased, judged by thyroid morphology and I^{131} uptake in hypophysectomized recipients (Table 2). Daily doses of 3 to 5 cc. of plasma increased the height of the epithelial cells, caused vacuolation of colloid, and more than doubled the I^{131} uptake. Similar doses of plasma from normal rats, pregnant or non-pregnant, did not give these evidences of thyroid stimulation.

The effect of hypophysectomy on the growth-promoting activity in plasma of pregnant rats as judged by the tibia test is shown in Table 3.[5] The growth promoting potency of plasma from hypophysectomized-pregnant rats was greater than in normal rats, being of the order of that in normal-pregnant rats (4). The slight increases in body weights observed in hypophysectomized recipients were not considered reliable evidence for increased growth-promoting content of plasma, due to the brevity of the injection period (4 days). A more definite increase in body weight, as well as an increase in tail length, was observed when plasma obtained from rats on day 16 or 20 of pregnancy was injected for 14 or 15 days (Table 4). The plasma from both normal-pregnant and hypophysectomized-pregnant rats increased body weight and tail length to about the same extent. The width of the cartilaginous disc of the tibia was likewise increased after this longer injection period, and to approximately the same extent in recipients of plasma from both types of donor. The plasma from normal (non-pregnant) rats caused a smaller though consistent increase in tail length and tibial cartilage width.

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[5] Serum from non-pregnant hypophysectomized rats was not injected simultaneously with these experimental groups, however, the serum of rats was assayed 55 to 70 days after hypophysectomy and showed no evidence of growth promoting potency. Doses of 1, 3 and 5.5 cc. of serum were injected daily for 4 days into groups of 4 rats each. The proximal epiphyseal disc of the tibia of controls averaged 165 μ and of injected 179, 174 and 172 μ, respectively.

TABLE 4. INFLUENCE OF HYPOPHYSECTOMY ON THE GROWTH PROMOTING ACTIVITY OF PLASMA OF PREGNANT RATS JUDGED BY BODY GROWTH

Doners of plasma				Hypophysectomized recipients					
Type	Day of pregnancy	Days P.O.	Days P.O.	Days inj.	Daily dose	Tibial cartilage width	Body weight increase	Tail length increase	Thymus weight
					cc.	micra	gm.	gm.	mg.
Normal Non-pregnant	—	—	45	14	3.0	173±6	4±1.2	2 ±0.1	123±10
Normal-Pregnant	20	—			3.0	208±6	8±1.0	5 ±0.3	152± 6
Hypophysectomized-Pregnant	20	8			3.5	221±6	6±0.8	5 ±0.3	134±10
	—	—			0	140±9	2±1.2	0.5±0.1	99± 7
Normal Non-Pregnant	—	—	30	15	3.0	175±5	11±1.0	3.5±0.2	150± 4
Normal-Pregnant	16	—			3.4	220±4	16±1.5	6.6±0.3	150± 4
Hypophysectomized-Pregnant	16	4			3.0	200±6	10±1.0	5.2±0.2	164±12
	—	—			0	143±3	0±0	0.7±0.1	99± 5

The weight of the thymus was increased somewhat by injections of plasma for both 4 and 14 days, especially by plasma from pregnant rats, either normal or hypophysectomized. No gonadotrophic or adrenocorticotrophic activity was detected in the plasma from any type of donor at the doses injected, as judged by ovarian weight, follicular growth and interstitial cell repair, or by adrenal weight and cortical lipid distribution.

DISCUSSION

The persistence of greater than normal amounts of growth-promoting activity in the plasma of pregnant rats after hypophysectomy indicates a source outside the maternal pituitary. Maternal pituitary growth hormone would have disappeared from the circulation long before the period when plasma was drawn for bioassay. The anticipated period of survival of growth hormone in the circulation after hypophysectomy is relatively short as far as can be judged from injection of growth hormone, which indicated a half-life of 8–10 hours (10, 11). An increased growth-promoting activity in plasma is here reported 4 to 8 days after hypophysectomy, that is, on days 16 to 20 of pregnancy. The fetal pituitary probably does not contribute growth hormone to the plasma in the earlier period of pregnancy (12, 13). In later pregnancy, day 20, the potency of plasma was not greater than on day 16, although it is known that the fetal pituitary by this period contains growth hormone (14). It seems probable therefore, as others have postulated previously (15), that the placenta is a source of growth-promoting substance in the hypophysectomized-pregnant rat.

After thyroidectomy of the non-pregnant rat the growth-promoting activity of plasma has been found to decrease parallel with the decrease in the pituitary growth hormone content (5, 6, 16), whereas after thyroidectomy of the pregnant rat there is no comparable decrease in growth promoting potency of the plasma. It seems probable therefore that either the origin of the growth-promoting substance or the mechanism of its release is different in the two cases. In thyroidectomized-pregnant rats the growth promoting activity present during the first half of pregnancy probably originates in the placenta, though conceivably also in the maternal pituitary. In later pregnancy the fetal pituitary may contribute.

The thyroid hormone of maternal origin does not appear to be a synergistic agent contributory to the increased growth potency of plasma of the pregnant rat. Thyroid function is actually reduced in the rat during normal pregnancy, as far as can be judged by the reduced I^{131} uptake by the thyroid. A reduction in protein bound iodine in plasma has also been reported in the rat during pregnancy (17). Furthermore, the growth-promoting activity of plasma of hypophysectomized-pregnant rats was found to be similar to that in normal pregnancy, whereas the thyroid function was reduced below that in intact-pregnant rats. The growth-promoting activity in the plasma of the thyroidectomized-pregnant rats was also similar to that in intact-pregnant rats in spite of the complete absence of thyroid tissue. Persistence in the circulation of maternal thyroxine seems unlikely, since the half-life of thyroxine is a matter of hours (18–21). At the time the plasma of thyroidectomized-pregnant rats was bioassayed and found to contain growth stimulating substances, 13 days after thyroidectomy, the amount of circulating thyroxine would therefore be insignificant. The activity of the fetal thyroid must be kept in mind in considering later pregnancy. The fetal thyroid is known to be able to accumulate iodine, and to produce and release thyroid hormone into the fetal circulation (22, 23). It is possible that fetal thyroxine may cross the placenta in amounts sufficient to synergize the growth-promoting activity of maternal plasma. Fetal thyroxine may also reinstate the growth hormone-producing acidophils of the maternal pituitary, as it is known that only small amounts of thyroxine are necessary for this effect (24, 25).

The thyrotrophic hormone content in plasma is known to increase in the non-pregnant thyroidectomized rat within the post-operative period in question and this rise occurs only when the thyroxine level is very low (24, 25). In the present examination of the plasma of thyroidectomized-pregnant rats, the thyrotrophic hormone content was found to be increased to approximately the same level as in non-pregnant thyroidectomized rats, indicating that here too the level of thyroxine was low. Both the morphology and I^{131} uptake of the thyroids of hypophysectomized-pregnant rats indicated that thyroids of these animals were also under the influence of a thyroid stimulating substance not present in the non-pregnant hypophysectomized rat. Such thyrotrophic substances could be contributed either by the placenta or the fetal pituitary. Thyrotrophic hormone has been demonstrated to be present in the fetal rat pituitary in later pregnancy (13). The amount secreted by the fetal rat pituitary has even been shown to be capable of increasing under stimulation, as thyroid hyperplasia has been observed in fetuses of hypophysectomized-thyroidectomized pregnant rats injected with goitrogens (26). It seems less probable that the placenta produces and releases thyrotrophic substances, since it has been shown that the thyroids of decapitated fetal rats did not become hyperplastic after injection of propylthiouracil (27). This leaves the fetal pituitary as the more probable source of thyrotrophin in the hypophysectomized-pregnant rat.

Evidence for the production of some other hormones beside growth and thyroid stimulating hormones was also recorded. Enlargement of the follicles and increased ovarian weight was noted on day 20 of pregnancy in normal rats. This enlargement was probably preparatory for ovulation which is known to occur at parturition (28). The resumption of secretion of pituitary follicle stimulating hormone has been assumed to account for this follicular growth and the accompanying ovarian weight increase. The ovaries of hypophysectomized-pregnant rats did not decrease in weight as occurs after hypophysectomy in the non-pregnant rat. The ovaries even increased in size slightly later in pregnancy. The corpora lutea remained large and the luteal cells were almost as large as in intact pregnant animals, but no follicular enlargement occurred. It appears therefore that in the absence of the maternal pituitary, secretion of gonadotrophins capable of stimulating follicular growth does not occur, but that the placenta contributes a substance which maintains the corpora lutea (29, 30). In non-pregnant thyroidectomized rats the ovaries regressed and ovulation rarely occurred, but the ovaries of thyroidectomized-pregnant rats increased in weight late in pregnancy and contained large follicles as well as large corpora lutea. From this it would appear that in the thyroidectomized-pregnant rat the pituitary or the placenta, or both organs, continue to secrete the necessary gonadotrophic substances. The pituitary of the thyroidectomized rat has been shown to be able under certain circumstances to produce and release an amount of gonadotrophin comparable to that of the normal pituitary. When, for example, gonadectomy was superimposed on thyroidectomy both the pituitary and plasma were found to contain greater amounts of gonadotrophins than after thyroidectomy alone (5, 16, 31).

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GASTRIC SECRETORY ACTIVITY IN MICE AND RATS WITH TRANSPLANTED HORMONE SECRETING ADRENOCORTICAL TUMORS[1]

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ABSTRACT

Abnormalities of gastric secretory activity have been reported in both hyper and hypo-adrenocorticoidism in man and animals. It has been suggested that different adrenocortical steroids vary in their effect upon gastric secretion.

Gastric secretory activity was studied in mice and rats with transplanted hormone secreting adrenocortical tumors, and in normal rats receiving corticosterone and desoxycorticosterone acetate. Corticosterone and 11β-OHΔ^4 androstene-3,17-dione are the principal steroids produced by the mouse adrenal tumor and corticosterone is the major steroid secreted by the rat adrenal tumor. No 17α hydroxylated adrenocortical steroids have been detected in the adrenal tumor tissues *in vivo* or *in vitro* in these animals.

There was no significant difference in gastric juice volume, pH, hydrochloric acid, potassium and chloride concentration or pepsin content in the tumor bearing animals when compared to the controls. The sodium content of the juice in tumor bearing rats was significantly elevated probably reflecting a mineralocorticoid effect. There was no difference in surface tension or rheological behavior. No pathological changes of the stomach wall were observed in either group.

Corticosterone or desoxycorticosterone acetate did not influence any parameter of gastric secretion in normal rats. These studies suggest that corticosteroids without 17α hydroxylation do not affect gastric secretory activity or induce gastric ulceration. This is in contrast to gastric secretory changes and ulceration reported in animals receiving 17α hydroxylated corticosteroids.

THE effect of adrenocortical activity upon gastric secretory function is illustrated by the abnormal gastric secretion described in clinical states of both hyper and hypo-adrenocorticoidism (1, 2, 3). Patients with

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the hypercorticoidism of Cushing's syndrome have been reported to secrete more gastric acid than normal after a gruel test meal (1). Patients with adrenal or pituitary over-activity (Cushing's disease) excreted abnormally high quantities of uropepsin (2, 3). Adrenocortical hyperplasia and tumors have been observed in association with multiple endocrine tumors accompanied by gastrointestinal ulceration (4).

On the other hand, adrenocortical hypofunction in man is associated with abnormally low quantities of gastric hydrochloric acid (2, 5), and peptic ulceration in Addison's disease has been observed infrequently (2, 3). Reduced gastric activity in adrenocortical insufficiency has been demonstrated in animals and man with restoration to normal levels by the administration of cortisone (2, 3, 6, 7, 8) but not by desoxycorticosterone acetate (DCA) (9). It has been suggested that cortisone-like steroids are those which affect gastric secretory activity, in contrast to corticosterone and desoxycorticosterone acetate which are without demonstrable effect (3, 10).

The development of transplantable hormone secreting adrenocortical tumors in mice and rats by Cohen *et al.* (11, 12) presented the opportunity to investigate the gastric secretory activity in these animals. Corticosterone and 11 β-OH-Δ^4 androstene-3,17-dione are the principal steroids produced by the mouse adrenal tumor, and corticosterone is the major steroid secreted by the rat adrenal tumor (12). No 17α hydroxylated adrenocortical steroids have been detected in the adrenal tumor tissues *in vivo or in vitro* (12, 13) in these animals.

MATERIALS AND METHOD

Hormone secreting adrenocortical tumors were transplanted into LAF_1 mice and into WR rats by the method previously described (11). Animals with tumor transplants and normal control animals of the same age and strain were maintained on a normal diet for six weeks in the case of the mice, and sixteen weeks in the case of the rats. At the end of this time all the mice and the majority of the rats with transplants had visible tumors. The remainder of the rats with transplants had palpable tumors.

The effect of desoxycorticosterone acetate (DCA) was evaluated by injecting intramuscularly into Sprague-Dawley female rats 2.5 mg. DCA daily for seven days. Corticosterone in doses of 2.5 mg. daily was injected intramuscularly for three days in one group and for seven days in another. A group of twenty-nine normal females were used as controls for these experiments.

Gastric juice was obtained for analysis from both mice and rats by the Shay procedure. In the rats the stomachs were removed four hours after pyloric ligation, and in the mice twelve hours after ligation. The juice was analyzed for volume, pH, hydrochloric acid, pepsin and electrolyte content as previously described (14). The significance of the difference between means was calculated using Student's "t" test and the 2% level of significance. The stomachs were examined macroscopically and microscopically.

The surface tension of gastric juice was measured using a Langmuir-Wilhelmy film balance as described by Avery *et al.* (15). Two cc. of gastric juice were diluted with 63 cc. of physiological saline for analysis. Since surface tension is a function of film area the tension was recorded at different areas, the surface being compressed to 15% of

TABLE 1. GASTRIC SECRETION IN MICE AND RATS WITH TRANSPLANTED HORMONE SECRETING ADRENOCORTICAL TUMORS

	Number of animals	Volume* (cc.)	pH	HCl		Na	K	Cl	Gastric† juice pepsin	Gastric‡ wall pepsin
				Free	Total	(m eq./l.)				
				(m.eq./l.)						
Normal mice	8	6 ±2.2	2.8 ±0.9	24 ±24	49 ±41	95 ±16	12 ± 5.5	186 ±12	21,690 ±22,000	655 ±345
Mice with tumors	12	7.1 ±1.4	2.3 ±0.6	25 ±11	56 ±30	107 ±16	10.3 ± 3.8	192 ±47	22,230 ± 6,700	671 ±780
Normal rats	7	1.2 ± .8	1.7 ±0.7	60 ±40	105 ±50	50 ± 4.2	16.6 ±11.0	156 ±11	231 ±180	1,290 ±114
Rats with tumors	14	0.85 ±0.2	2.5 ±1.3	35 ±27	84 ±25	74 ±21	12.5 ± 6.4	146 ±16	116 ± 31	1,387 ±335

Mean values with standard deviations.
* Cc. per 100 gm. body weight.
† Peptic units/cc./100 gm. body weight ×volume secreted.
‡ *Peptic units/100 gm. body weight in whole stomach.*

its original area. The rheological behavior was studied in a cone-and-plate type of viscometer at 37° C using shear rates of 12, 30 and 60 r.p.m. and 3 cc. centrifuged samples, pooled where necessary (16).

RESULTS

The gastric secretory activity in mice and rats with transplanted adrenocortical tumors (secreting predominantly corticosterone) is compared with control animals in Table 1. There was no statistically significant difference in volume, pH, hydrochloric acid, potassium and chloride concentration, and pepsin content of gastric juice in tumor bearing animals when compared with controls. Similarly, the pepsin content of the stomach wall was not different in the two groups. The sodium content of the juice in tumor bearing rats was significantly higher than in the control animals probably reflecting a mineralocorticoid effect. There was no change observed in volume, pH, hydrochloric acid, sodium, potassium and pepsin content of the gastric juice after the injection of corticosterone or desoxycorticosterone acetate into normal rats (Table 2).

The physical properties of gastric juice were evaluated by surface tension and viscosity measurements. Measurements of the surface tension as

TABLE 2. THE EFFECT OF CORTICOSTERONE AND DESOXYCORTICOSTERONE UPON THE GASTRIC SECRETION OF THE RAT

	Number of animals	Volume* (cc.)	pH	HCl		Na	K	Gastric† juice pepsin
				Free	Total	(m.eq./l.)		
				(m.eq./l.)				
Controls	29	3.8±1.4	1.26±0.17	93±22	127±21	33.4±21	9.3±3.6	770±340
Corticosterone (3 days)	8	2.7±0.3	1.3 ±0.1	100±10	130±20	24 ± 8	10.6±2.1	517±140
Corticosterone (10 days)	7	3.4±0.6	1.1 ±0.1	86±20	119±18	34 ±13	10.7±5	659±160
Desoxycorticosterone Acetate (7 days)	10	3.2±1.2	1.1 ±0.2	98±13	117±13	45 ±18	9.6±2.5	594±240

* Cc. per 100 gm. body weight.
† Peptic units/cc./100 gm. body weight ×volume secreted.

TABLE 3. SURFACE TENSION IN DYNES/CM. OF GASTRIC JUICE FROM RATS BEARING HORMONE SECRETING ADRENOCORTICAL TUMORS AND NORMALS

Relative area (%)	Gastric juice from rats bearing tumors			Gastric juice from normal rats	
	1	2	3	4	5
	(dynes/cm.)			(dynes/cm.)	
100	43.6	41.0	40.5	39.0	
90	36.8	36.8	35.5	34.7	41.7
80	34.8	35.0	34.2	34.3	36.8
70	34.8	34.0	34.2	32.5	34.4
60	33.0	34.0	33.0	32.0	30.7
50	33.0	33.0	32.0	29.5	28.2
40	30.5	31.8	29.5	28.2	27.0
30	29.5	30.5	27.0	24.5	25.8
20	27.0	27.5	25.0	23.2	23.3
15	27.0	25.7	24.5	22.0	22.0
20	44.0	38.6	41.5	38.0	46.6
30	49.0	42.8	44.5	42.0	
100	60.0	58.8	57.5	57.5	58.9

a function of area revealed no significant difference in the juice obtained from tumor animals and controls (Table 3). Viscosity measurements at different shear rates (Table 4) showed the rheological behavior of gastric juice of tumor animals was similar to that of controls.

Macroscopic mucosal ulceration was not seen in any of the animals in this study, and histological examination revealed no difference in the mucosa from tumor bearing animals when compared with normals.

DISCUSSION

The absence of significant changes in gastric secretory function in mice and rats with hormone secreting adrenocortical tumors (Table 1) may be attributed to the lack of 17α hydroxylated corticosteroids which have been reported to increase gastric secretory activity and acid production in animals (17, 23). The 17α hydroxylated adrenocortical steroids have not been found in the tumor bearing animals (12, 13) or in normal mice and rats in significant quantity (12, 13, 18, 19, 20, 21).

Corticosterone appears to be the predominating active steroid in both the tumor bearing animal and normal mice and rats (12, 13, 18, 19, 20, 21). The unaltered gastric secretion observed in the tumor bearing animals and

TABLE 4. THE RHEOLOGICAL BEHAVIOR OF GASTRIC JUICE FROM NORMAL MICE AND RATS AND FROM ANIMALS BEARING HORMONE SECRETING ADRENOCORTICAL TUMORS

Shear rate in r.p.m.	12	30	60
Gastric juice from tumor rat	0	5.0	10.0
Gastric juice from normal rat	1.0	6.0	9.5
Gastric juice from tumor mice	2.5	4.0	10.0
Gastric juice from normal mice	6.0	8.0	14.0

The units of measurement at different rates of shear are arbitrary and are proportional to the absolute viscosity. Note the increased deflection with increasing shear rate is similar in tumor bearing animals and controls indicating there is no significant difference in the behavior of gastric juice from the two groups.

in the normal rats receiving corticosterone and desoxycorticosterone acetate support previous findings in humans that these corticosteroids are without significant effect upon gastric secretion (2, 3). Oral corticosterone did not affect the uropepsin/creatinine ratio in humans while the 17α hydroxylated compound, hydrocortisone, increased gastric secretory activity as measured by this ratio (22). Similarly, the 17α hydroxylated adrenocortical steroids have been reported to increase gastric secretion in humans (3) and in animals (17, 23). Moreover, depressed gastric secretion in humans with adrenocortical insufficiency may be restored by cortisone administration (2, 8).

Gastric mucin, important for gastric mucosal defense, influences the physical properties of gastric juice. Both surface tension phenomena and the rheological behavior of gastric juice from tumor bearing animals and normals were the same, suggesting that under the conditions of these experiments there was no demonstrable qualitative effect on gastric mucin. Decreased gastric juice viscosity has been reported following the administration of ACTH to humans (24). Reduction in the quantity of gastric mucin has been observed histologically after cortisone and hydrocortisone (6).

The gastric mucosa in animals subjected to increased endogenous or exogenous corticosterone or exogenous desoxycorticosterone acetate in this study was free of ulceration and essentially normal both macroscopically and microscopically. This is in contrast to reports that rats given the 17α hydroxylated adrenal steroids regularly develop gastric ulceration (7, 26).

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THE EFFECT OF HYPOPHYSECTOMY AND OF TSH ON THE MOUSE SUBMAXILLARY IODIDE PUMP[1]

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ABSTRACT

The I^{131} that is concentrated by the submaxillary glands of mice injected with radioiodide remains in the form of inorganic iodide, as revealed by paper chromatography and paper electrophoresis. The iodine concentrating capacity of submaxillary gland tissue in three strains of mice was determined by measuring the ratio,

$$\frac{I^{131}\text{ concentration in submaxillary}}{\text{radioiodide concentration in plasma}}$$

(S/P ratio), at various intervals after I^{131} injection. S/P ratios ranged from values of 4 to 7 for C_3H and Webster-Swiss mice to values of more than 30 for Cal A mice. In the former groups the maximum ratio was reached in about one hour, in the latter, after 4 hours.

Hypophysectomy and TSH injections did not induce any significant change in S/P ratios although, in the same mice, thyroid I^{131} uptakes or T/S ratios were markedly affected. It is concluded that the submaxillary iodide pump, unlike the thyroid iodide pump, is not directly affected by the presence or absence of TSH. These observations are discussed in relation to a previously proposed mechanism of action of TSH on the thyroid gland.

IT HAS become increasingly evident in recent years that the thyroid gland is not the only mammalian tissue which can concentrate inorganic iodide. Other tissues, such as gastric mucosa (1–6), salivary glands (2, 4, 7), lactating mammary gland (4, 8), and placenta (9, 10) have also been shown to possess mechanisms for transporting iodide against a concentration gradient. These extrathyroidal iodide pumps, like that of the thyroid, are inhibited by thiocyanate and perchlorate, a findings which led Rowlands and his coworkers (11, 12) to postulate an iodide transport mechanism for salivary tissue similar to that of the thyroid. Logothetopoulos and Myant (13) also concluded, from their radioautographic studies of the localization of iodide-I^{131} and S^{35}-labeled thiocyanate in thyroid, salivary glands and gastric mucosa, that iodide transport in these tissues operates via a common mechanism.

Since it is well known (14–16) that the thyroid iodide pump is largely under the control of pituitary thyrotropic hormone (TSH), it seemed of

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TABLE 1. SUBMAXILLARY AND THYROID I^{131} IN CAL A STRAIN MICE AT INTERVALS AFTER I^{131} INJECTION

Exp	No. of mice per group	Age	Diet	Interval after I^{131}	Mean body wt.	Mean submaxillary wt.	Mean thyroid wt.	Submaxillary I^{131}			Thyroid I^{131}			S/P
								% of injected I^{131}	Distribution within gland		% of injected I^{131}	Distribution within gland		
									Iodide	Non-iodide		Iodide	Non-iodide	
				hr.	gm	mg	mg.		%	%		%	%	mean
1	2	18 mos.	Purina Lab. Chow	0.5	32.8	179	5.6	5.3	99.8	0.2	1.3	29.0	71.0	5.5
	2			2	28.5	177	5.3	11.6	99.8	0.2	4.0	10.6	89.4	14.9
	2			4	28.8	160	5.0	15.0	99.9	0.1	6.3	7.0	93.0	32.8
	2			7.25	30.2	180	4.1	5.1	99.8	0.2	—	—	—	29.5
2	4	18 mos.	Purina Lab. Chow	1	24.6	163	—	10.2	—	—	2.8	—	—	12.9
	4			2	29.6	192	—	13.8	—	—	6.5	—	—	20.1
	4			4	23.8	133	—	17.4	—	—	8.2	—	—	33.5
3	2	3 mos.	Purina Lab. Chow	2	24.4	144	—	3.3	99.9	0.1	—	—	—	8.8
	2			4	23.5	142	—	4.4	99.9	0.1	—	—	—	16.5
	2			7	22.0	118	—	1.6	99.9	0.1	—	—	—	27.8
4	4	56 days	Diet 1 (no added KI)	2	24.3	90	—	2.1	—	—	12.4	—	—	6.8
	5			4	24.2	93	—	4.9	—	—	22.6	—	—	15.9

interest to determine whether extrathyroidal iodide transporting mechanisms are subject to similar control. A convenient means of investigating this question was suggested by the demonstration (12, 17) that mouse submaxillary tissue possesses a potent iodide concentrating mechanism, both *in vivo* and *in vitro*. In the present communication, we report our findings on the effects of hypophysectomy and of TSH on the mouse submaxillary iodide pump.

METHODS

Animals—Male mice of various strains and ages were used: 1) *Cal A Strain*—Four experiments were carried out with mice of this strain. The ages and diets of the various groups are shown in Table 1. 2) *C_3H Mice*—Most of the experiments involving hypophysectomy and TSH injection were performed on mice of this strain. These animals were received at approximately 35 days of age, and placed on a diet of the following composition (Diet 1): 67.5 per cent wheat, 15 per cent casein, 10 per cent whole milk powder, 5 per cent hydrogenated vegetable oil, 1 per cent NaCl, 1.5 per cent chalk, and 50 ml. of a vitamin A- and D-containing fish oil per 8 kg. of diet. This diet is low in iodine (about 0.2 μg. I/gm.). In some experiments (Table 2) the same diet was fed with sufficient added KI to bring the level up to 1 μg. I/gm. Hypophysectomies were usually performed 1 to 2 weeks after the mice had been received, and the animals were maintained on Diet 1 throughout the experiment. 3) *Webster-Swiss Mice*—A single experiment involving two different age groups was performed (Table 3). These animals received Diet 1 with KI supplement, beginning two days before hypophysectomy.

Technique of Hypophysectomy—Hypophysectomies were performed by Mr. C. Jordan according to the technique described by Lostroh and Jordan (18). The operative mortality rate varied in different experiments. It was greatest with the young Webster-Swiss mice (50 per cent), and least with the C_3H mice (0 per cent in one experiment). Sella turcica checks were performed at autopsy with the aid of the dissecting microscope. All hypophysectomies were complete as judged by this criterion. Seven to 10 days were allowed to elapse after hypophysectomy before the mice were injected with I^{131}.

I^{131}-Injection—Oak Ridge I^{131} was purified by distillation (19). Mice received 2 to 50 μc. of distilled I^{131}, including approximately 0.3 μg. of carrier iodide. Injections were

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON UPTAKE OF I^{131} BY SUBMAXILLARIES AND THYROIDS OF C_3H MICE

Exp.	Age	Treatment	No. of mice in group	Interval after I^{131}	Body weight	Submaxillary weight	Thyroid weight	Per cent of injected I^{131}		S/P
								Submaxillary	Thyroid	
	days			hr.	gm.	mg.	mg.			
1	42	Control	5	4–5	19.3±0.56†	66± 1.3		1.5±.13	3.8 ±.43	6.5±0.12
		HypX	6	4–5	14.7±0.69	47± 4.8		3.3±.37	0.40±.024	13.8±1.7
2	57	Control	6	2	20.3±1.0	89± 3.9	1.3±.052	2.7±.35*	2.5 ±.24	4.6±0.36
		HypX	4	2	16.7±0.96	54± 4.8	1.3±.060	2.5±.32*	0.19±.017	6.2±0.90
3	55	Control	5	1	21.2±1.3	82± 5.3	1.8±.035	3.9±.52*	1.8 ±.084	7.6±0.86
		HypX	5	1	20.5±0.42	53± 2.1	1.3±.14	3.1±.31*	0.20±.017	7.9±0.83
		TSH	8	1	22.0±0.65	94± 3.3	3.1±.11	2.8±.21*	5.0 ±.33	6.2±0.57
		Control	5	4	21.6±1.3	84±10.8	1.6±.17	5.4±.94*	5.2 ±.54	11.4±1.3
		HypX	5	4	17.5±0.79	52± 3.1	1.0±.032	4.2±.70*	0.17±.016	9.4±2.0

* Paper chromatography was performed on these samples, and only a band for iodide-I^{131} was observed on the radioautograms.

† Mean ± standard error. S.E. = $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$

usually given intraperitoneally, and the mice were sacrificed from 30 minutes to 24 hours later.

Treatment of Tissues—At the conclusion of the experiment, the mice were anesthetized with Nembutal. Blood was taken directly from the heart with the chest open, and the thyroid and submaxillary glands were removed, the former with the aid of a dissecting microscope. Thyroids were rapidly weighed on a previously tared microscope slide on a semi-micro analytical balance; submaxillary glands were weighed on a torsion balance to the nearest mg.

In those experiments in which the nature of the I^{131} in the submaxillary glands was to be determined by filter paper chromatography or by paper electrophoresis, the tissue was first homogenized with bicarbonate buffer (6 to 10 vol.) containing approximately 0.001*M* thiouracil, and 20 μl. of the homogenates were applied to the filter paper strips. Chromatography was carried out in collidine-water-ammonia, and in butanol-ethanol-2N NH_4OH (20). Paper electrophoresis was performed at pH 8.3 in 0.06M veronal buffer, and at pH 6.2 in 0.05M citrate buffer.

TSH Treatment—Parke, Davis and Co. TSH (Lot 50P4) was injected into both normal and hypophysectomized mice. C_3H mice (Table 2 and Fig. (4) received subcutane-

TABLE 3. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON UPTAKE OF I^{131} BY SUBMAXILLARIES AND THYROIDS OF WEBSTER-SWISS MICE

Age	Treatment	No. of mice in group	Interval after I^{131}	Body weight	Submaxillary weight	Thyroid weight	Per cent of injected I^{131}		S/P
							Submaxillary	Thyroid	
			hr.	gm.	mg.	mg.			
55 days	Control	5	1	19.9±0.81*	78±3.7	1.8±.16	1.3 ±.24	1.0 ±0.21	4.4±0.63
	HypX	3	1	15.8±0.15	47±6.6	1.1±.017	2.1 ±.17	0.13±0.046	5.2±0.17
	TSH	6	1	19.3 0.68	85±5.3	3.4±.16	0.95±.030	8.3 ±1.1	2.7±0.26
	Control	5	4	21.1±0.29	110±2.7	1.8±.046	0.69±.18	2.2 ±0.48	3.2±0.54
	HypX	4	4	16.8±0.36	56±4.1	1.3±.17	1.5 ±.47	0.12±0.035	9.3±1.9
6 mos.	Control	5	1	31.8±1.8	196±7.1	3.3±.29	2.3 ±.27	1.5 ±0.13	3.8±0.54
	HypX	4	1	26.1±0.36	98±8.7	2.7±.25	4.3 ±.68	0.25±0.032	7.0±1.2
	Control	5	4	30.6±0.79	172±8.4	4.1±.39	1.1 ±.29	2.9 ±0.43	5.2±0.66
	HypX	5	4	27.0±1.3	89±7.3	3.1±.36	4.5 ±.93	0.15±0.035	18.0±1.7

* Mean ± standard error. S.E. = $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$

ous injections twice daily, each injection consisting of 0.5 mg. of TSH in 0.1 ml. isotonic saline. Webster-Swiss mice (Table 3) received injections of 1 mg. of TSH in 0.1 ml. saline twice daily. Injections were administered over a period of three days, a single TSH injection being given on the fourth day (7 injections in all). I^{131} was administered 1 to 5 hours after the last TSH injection.

Calculation of S/P Ratios—The I^{131} concentrated by mouse submaxillary tissue was present only in the form of inorganic iodide (see below). Furthermore, at early intervals after I^{131} injection, the I^{131} in plasma was also present primarily as inorganic iodide. Even in normal animals maintained on Diet 1 without added iodide, protein-bound I^{131} in plasma comprised less than 5 per cent of the total I^{131} 4 to 5 hours after I^{131} injection, as determined by precipitation with trichloroacetic acid. In the plasma of normal mice maintained on Purina laboratory chow, there was little evidence of organic I^{131} in plasma even 7 hours after I^{131} injection, as determined by filter paper chromatography. Therefore, in mice on Diet 1 sacrificed up to 4.5 hours after I^{131} injection, and in mice on Purina laboratory chow, sacrificed up to 7 hours after I^{131} injection, the ratio

$$\frac{\text{total } I^{131} \text{ per mg. of submaxillary tissue}}{\text{total } I^{131} \text{ per } \mu\text{l. of plasma}}$$

(S/P ratio) was taken as a measure of the degree to which submaxillary tissue is able to concentrate iodide.

In normal mice treated with TSH, analysis of plasma by filter paper chromatography revealed that approximately 95 per cent of the I^{131} in plasma was present as inorganic iodide 2 hours after I^{131} injection, and about 80 per cent 4 hours after I^{131} injection. In a group of C_3H mice sacrificed 24 hours after I^{131} injection (Fig. 3), 30 to 70 per cent of the plasma I^{131} was inorganic. In all of these groups, S/P ratios were based on the measured inorganic I^{131} concentration of plasma rather than on the total I^{131} concentration.

S/P Ratios in Mice Treated with Methyl Mercaptoimidazole—It seemed of interest to compare, in the same group of mice, the effect of TSH on the submaxillary iodide trap with its effect on the thyroid iodide trap. To facilitate measurement of the latter, mice were first injected with methyl mercaptoimidazole (MMI) which, in preliminary experiments, was found to block organic iodine formation in the thyroid completely at a dose of 10 mg. per 100 gm. body weight.

C_3H mice, 56 days old, that had been maintained on Diet 1 without added iodide for three weeks, were divided into four groups: normal, normal injected with TSH, hypophysectomized, and hypophysectomized injected with TSH. TSH injections were exactly as described above. Two to three hours after the last TSH injection, and nine days after hypophysectomy, each mouse received an intraperitoneal injection of MMI (10 mg. per 100 gm. body weight) and, 30 minutes later, an injection of I^{131} containing 0.3 μg. I^{127}. Animals were anesthetized with Nembutal 90 minutes after the I^{131} injection, blood was withdrawn from the heart, and thyroid and submaxillary glands were removed. S/P and T/P

$$\left(\frac{\text{thyroid } I^{131} \text{ concentration}}{\text{plasma } I^{131} \text{ concentration}}\right)$$

ratios were determined.

RESULTS

Nature of I^{131} Concentrated in Mouse Submaxillary Gland—Figure 1 illustrates paper chromatographic results obtained with submaxillary tissue from C_3H mice two hours after I^{131} injection. Results in two different chromatographing solvents are shown, and in each case, only a single

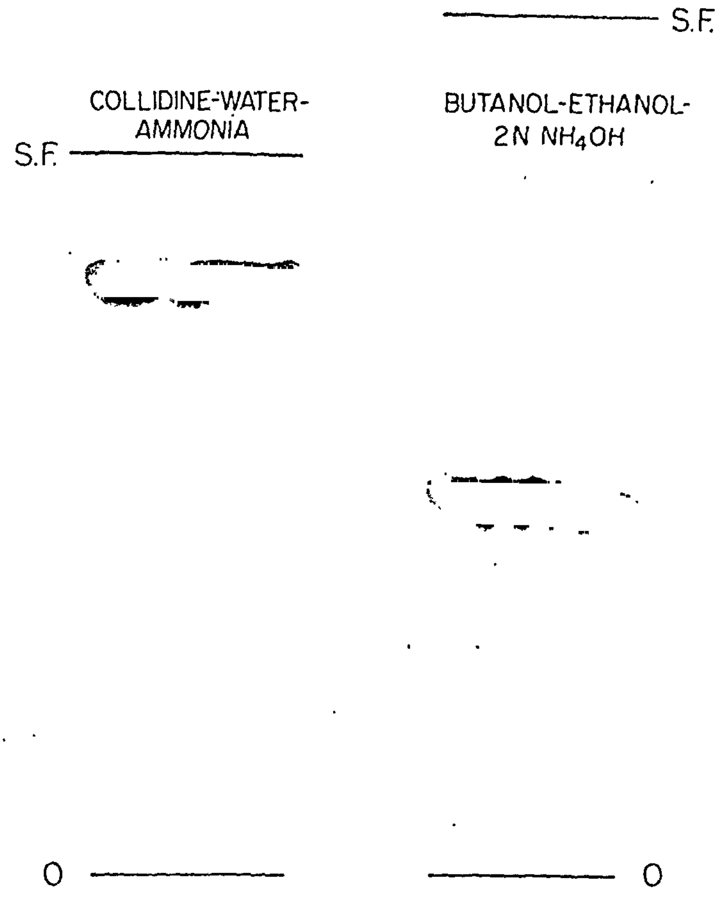

FIG. 1. Radioautographs of filter paper chromatograms of mouse submaxillary gland tissue 2 hours after injection of radioiodide. O, origin; S.F., volvent front. Inorganic I^{131} was the only component visible in the two different solvents indicated.

component was detectable on the radioautographs—that corresponding to inorganic iodide. Similar results were obtained in many other experiments, with both Cal A and C_3H mice, and at intervals from ½ to seven hours after I^{131} injection.

Paper electrophoresis, too, was employed to examine the chemical nature of the I^{131} concentrated by mouse submaxillary gland tissue. As revealed by this procedure also, iodide-I^{131} was the only radioactive component present. Typical results are ilustrated in Figure 2, which shows the electrophoretic pattern obtained at pH 8.3 and at pH 6.2, two hours after I^{131} injection.

In no case was there any evidence of the unknown compound (U-2) which we previously encountered when cell-free preparations of rat submaxillary gland tissue were incubated with iodide-I^{131} *in vitro* (21).

Normal S/P Ratios—The highest values for S/P ratios obtained in the present study were observed in mice of the Cal A strain. As shown in Table

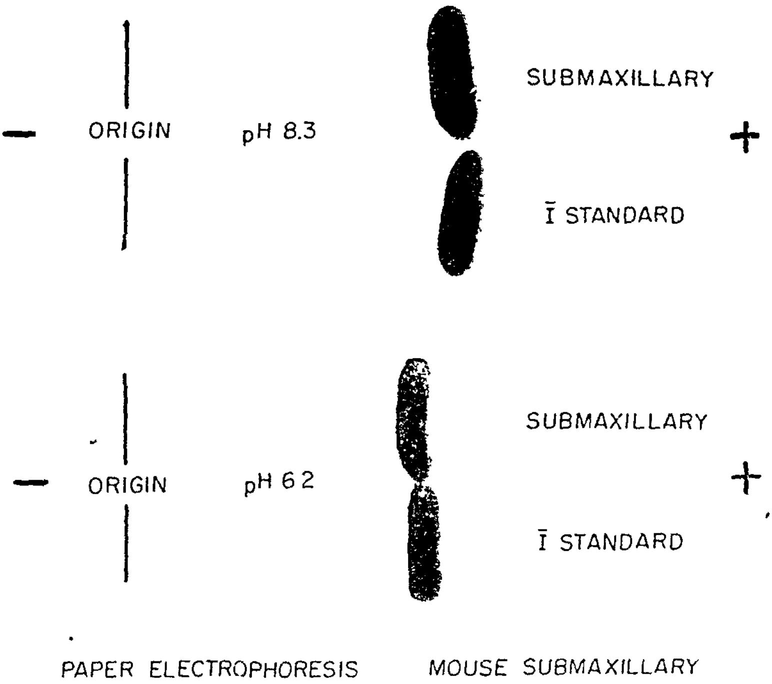

FIG. 2. Radioautographs of paper electrophoretigrams of mouse submaxillary tissue 2 hours after injection of radioiodide. Conditions of electrophoresis: Whatman No. 1 filter paper, 490 volts, 5 ma, 60 minutes duration. Buffers used: pH 8.3, 0.05M Veronal buffer; pH 6.2, 0.06M citrate buffer. Inorganic I^{131} was the only component visible at both pH's.

1, values greater than 30 were obtained in some experiments. S/P ratios in these animals appeared to increase with time, reaching a maximum value 4 to 7 hours after I^{131} injection.

With C_3H and Webster-Swiss mice, on the other hand, S/P ratios were considerably lower, and there was no consistent increase with time following the 1-hour measurement. This is clearly shown for C_3H mice in Figure 3 in which S/P ratios are plotted against time, from 1 hour to 25 hours. The mean value of 7.8 obtained for S/P at 1 hour was not significantly different from the mean value of 6.7, obtained at 24 hours. Values for S/P ratios in Webster-Swiss mice, shown in Table 3, ranged from 3.2 to 5.2, and did not change significantly between 1 and 4 hours.

S/P Ratios after Hypophysectomy—The iodide concentrating capacity of submaxillary gland tissue, as measured by the S/P ratio, was not impaired by hypophysectomy. This is shown by the data in tables 2 and 3.

Table 2 illustrates results obtained with C_3H mice in several different experiments. In no case was the S/P ratio significantly decreased after

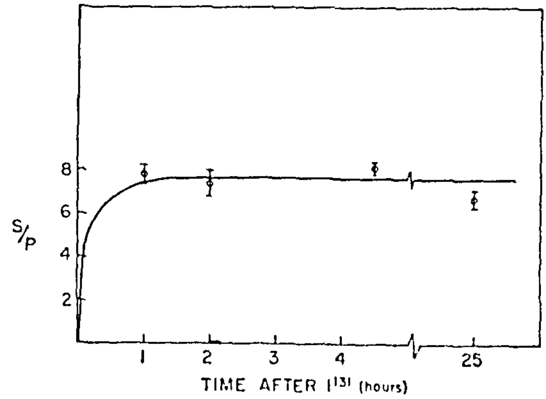

FIG. 3. Curve showing change in S/P ratio with time after I^{131} injection in normal C_3H mice. Each point represents the mean of results obtained with 4 or 5 animals. Vertical lines passing through points indicate standard error of the mean.

hypophysectomy, although thyroid uptakes of I^{131} in the same animals were greatly reduced. Results of similar experiments with Webster-Swiss mice are shown in Table 3. In the latter, an increase in S/P was usually observed after hypophysectomy. Possible reasons for this increase are discussed below.

S/P Ratios in TSH-Injected Mice—Injections of TSH into either normal or hypophysectomized mice had no significant effect on S/P ratios. This is illustrated by Experiment 3, Table 2, in which administration of TSH to normal C_3H mice had no effect on S/P ratios, although thyroid weight and thyroid uptake of I^{131} were markedly increased. Similar observations are recorded for Webster-Swiss mice in Table 3.

In Figure 4, effects of TSH injection on both S/P ratios and thyroid I^{131} uptakes are compared in normal and in hypophysectomized C_3H mice at various intervals after I^{131} injection. The effect of TSH administration on thyroid I^{131} uptake is immediately apparent, but the salivary iodide concentrating mechanism in the same animals was not affected by treatment with the trophic hormone.

S/P and T/P Ratios in Mice Treated With Mercaptoimidazole—Thyroid and submaxillary iodide traps were compared in four groups of mice, as described above under "Methods." Organic binding of I^{131} was blocked in these animals by methylmercaptoimidazole (MMI). The results, summarized in Table 4, show that, under these conditions also, hypophysectomy had no effect on S/P ratios. T/P ratios, however, were reduced by hypophysectomy to approximately $\frac{1}{5}$ the control value, a result in agree-

TABLE 4. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON S/P AND T/P RATIOS IN C_3H MICE TREATED ACUTELY WITH METHYLMERCAPTOIMIDAZOLE

Treatment	Number of mice in group	Body weight	Submaxillary weight	Thyroid weight	S/P	T/P
Normal controls	7	22.1 ± .73	70 ± 4.8	1.41 ± .050	20.5 ± 2.7	350 ± 28
HypX	7	18.4 ± .84	39 ± 2.1	1.16 ± .058	19.6 ± 3.6	76 ± 12
Normal + TSH	6	22.1 ± .66	78 ± 6.3	2.84 ± .056	14.0 ± 1.1	142 ± 10
HypX + TSH	7	15.7 ± .99	37 ± 5.0	1.70 ± .11	20.6 ± 1.7	156 ± 17

ment with observations previously made by Wollman and Scow (16). Administration of TSH to hypophysectomized mice partially restored T/P ratios, but had no significant effect on S/P ratios. The interesting finding that administration of TSH to normal mice significantly reduced T/P ratios agrees with the previous report of Scow and Greer (22) who observed a similar reduction in mice fed propylthiouracil (PTU). A possible explanation was provided by Halmi (6) who postulated that if "iodide acceptor sites" in the thyroid are already near a maximum, they may not be increased by excess TSH, whereas this hormone might, at the same time, still exert its usual effect on the weight of the gland.

A surprising result encountered in the experiment of Table 4 was the unexpectedly high value for S/P. The mean value was 20.5, more than twice the value obtained for similar mice not treated with MMI (Fig. 3). This raised the question whether MMI had some specific effect on the submaxillary iodide trap, and a separate experiment was performed to compare the effect of MMI with that of PTU on S/P and T/P ratios. Normal C_3H mice of the same age and maintained on the same diet as those employed above were divided into four groups: controls, saline-injected, MMI-injected and PTU-injected. MMI and PTU were given in approximately equimolar dosage, the former at 10 mg. per 100 gm. body weight, the latter at 15 mg. per 100 gm. body weight. I^{131} containing 0.3 μg. of stable iodide was injected 30 minutes after the saline or antithyroid drug,

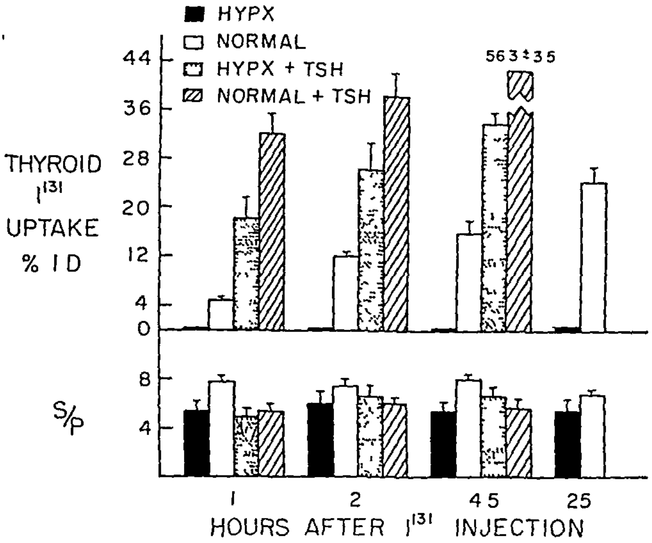

FIG. 4. Bar graph showing comparative effect of TSH administration on S/P ratios (lower section) and thyroid I^{131} uptakes (upper section) in normal and hypophysectomized C_3H mice. Each bar represents the mean of measurements made on 4 or 5 animals. The vertical line above each bar indicates the positive portion of the standard error of the mean.

TABLE 5. COMPARISON OF EFFECT OF METHYLMERCAPTOIMIDAZOLE AND PROPYLTHIOURACIL ON S/P AND T/P RATIOS IN NORMAL C_3H MICE

Treatment	Number of mice in group	Body weight	Submaxillary weight	Thyroid weight	S/P	T/P
None	6	21.3 ± .58	62 ± 2.6	1.27 ± .10	6.2 ± .52	
Saline	6	21.3 ± .81	58 ± 4.0	1.28 ± .062	6.6 ± .77	
MMI	6	21.7 ± .71	61 ± 1.8	1.31 ± .11	13.2 ± .60	315 ± 49
PTU	6	21.7 ± .71	63 ± 2.6	1.48 ± .13	6.3 ± .42	283 ± 35

and the animals were sacrificed one hour later. As shown in Table 5, S/P ratios in MMI-treated mice were again significantly greater than in control mice, whereas injection of PTU, or of saline alone, had no effect. T/P ratios in the MMI-treated mice, on the other hand, were not significantly different from those of PTU-treated mice. This observation suggests that MMI has some specific effect on mouse submaxillary gland tissue not shared by PTU. Further experiments are necessary to establish the mechanism of this effect.

Effect of Hypophysectomy on Submaxillary and Thyroid Weights—In mice deprived of their pituitaries, body, thyroid, and submaxillary weights were significantly reduced below control values (tables 2 and 3). Relative weights (i.e., weights per 100 gm. body weight) of thyroid and submaxillary were calculated for C_3H mice in several of the groups studied here, and the combined results are summarized in Table 6. Although absolute thyroid weights declined significantly after hypophysectomy, it is evident from Table 6 that there was no significant change in the relative weight of the thyroid. However, it was found that hypophysectomy resulted in a significant reduction in the relative weight of the submaxillary gland. This reduction was not reversed by administration of TSH either to normal or to hypophysectomized mice. Thyroid weight, on the other hand, did increase significantly in both groups treated with TSH.

Loss in relative weight of submaxillary tissue after hypophysectomy was previously noted in the mouse by Lacassagne and Chamorro (23), and in the rat by Gabe (24) and by Baker *et al.* (25, 26). In both species the principal site of regression was the serous tubules. Hypophyseal control

TABLE 6. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON THYROID AND SUBMAXILLARY WEIGHTS OF C_3H MICE

Treatment	Number of mice in group	Body weight	Submaxillary weight	Thyroid weight
			mg. per 100 gm. body wt.	mg. per 100 gm. body wt.
Normal controls	26	19.7 ± .56	326 ± 28	6.3 ± .17
HypX	27	15.7 ± .51	233 ± 22	6.5 ± .16
Normal + TSH	19	20.0 ± .56	352 ± 37	12.9 ± .43
HypX + TSH	19	15.0 ± .56	228 ± 32	10.6 ± .52